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Sequence-specific gene silencing by transitive signals in plants

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LIST OF ABBREVIATIONS

3'chs	3'-untranslated region of the chalcone synthase gene of *Antirrhinum*
3'g7	3'-untranslated region of the *A. tumefaciens* octopine T-DNA gene 7
3'nos	3'-untranslated region of the nopaline synthase gene
3'ocs	3'-untranslated region of the octopine synthase gene
3'-UTR	3'-untranslated region
asRNA	antisense RNA
bar	bialaphos acetyltransferase
bp	basepairs
CAT	catalase
CMT3	CHROMOMETHYLASE 3
DDM1	DECREASE IN DNA METHYLATION 1
DNA	deoxyribonucleic acid
DRM	DOMAINS REARRANGED METHYLTRANSFERASE
dsRNA	double-stranded RNA
EGO	enhancer of glp-1
ERI	enhanced RNAi
ESM	enhanced silencing movement
EtBr	ethidium bromide
GFP	green fluorescent protein
GUS	β-glucuronidase
H3K9	histone 3 lysine 9
HDA6	hisotone actetyltransferase 6
hpRNA	hairpin RNA
hpt	hygromycin phosphotransferase
HST	HASTY
IR	inverted repeat
Kb	kilobases
KYP	kryptonite
LB	left T-DNA border
MBD6	methyl binding protein 6
MET1	METHYLTRANSFERASE 1
miRNA	microRNA
M_r	molecular weight
mRNA	messenger RNA
nptII	neomycin phosphotransferase II

nt	nucleotides
P35S	cauliflower mosaic virus 35S promoter
PHB	PHABULOSA
PHV	PHAVULOTA
Pnos	nopaline synthase promoter
Pss	promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase
PSTVd	potato spindle tuber viroid
PTGS	post-transcriptional gene silencing
PVX	potato virus X
QDE	quelling deficient
RB	right T-DNA border
RdDM	RNA-directed DNA methylation
RDE	RNAi deficient
RDR	RNA-dependent RNA polymerase
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing
RNA	ribonucleic acid
RNAi	RNA interference
RRF	RdRP family
RRP	RNA-directed RNA polymerase
SD	standard deviation
SDE	silencing defective
SGS	suppressor of gene silencing
siRNA	small interfering RNA
SMD	silencing movement deficient
smRNA	small RNA
ssRNA	single-stranded RNA
SUVH	Su(var)3-9 homolog
SVISS	satellite virus-induced silencing system
TAS loci	trans-acting siRNA loci
ta-smRNA	trans-acting small RNA
TGS	transcriptional gene silencing
TSP	total soluble protein
VdIRS	viroid-induced RNA silencing system
VIGS	virus-induced gene silencing

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Chapter 1:

General introduction

Adapted and extended from:

Bleys, A., Van Houdt, H. and Depicker, A. (2006) Transitive and systemic RNA silencing: both involving an RNA amplification mechanism? In *Small RNAs: Analysis and Regulatory Functions.* (Nellen, W. and Hammann, C. eds.). Nucleic Acids and Molecular Biology, Vol. 17, Springer, Berlin, Germany, pp. 119-139.

1. The phenomenon of RNA silencing

1.1. The discovery of RNA silencing

In 1990, plant scientists unintentionally stumbled across an intriguing phenomenon. The introduction of transgenic DNA resulted not always in the expected overexpression phenotype, but some plants exhibited coordinated inhibition of the expression of both the transgene(s) and the homologous endogene, which was designated co-suppression at the time (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Details about the process behind this phenomenon were uncovered step by step, but two key findings, namely the discovery that double-stranded RNA (dsRNA) is a strong inducer of RNA silencing in *Caenorhabditis elegans* (Fire *et al.*, 1998), and the detection of small RNAs (smRNAs), 21-26 nucleotides (nt) in size, in plants (Hamilton and Baulcombe, 1999), have speeded up the elucidation of the mechanism of RNA silencing (see Section 1.3). Nowadays, the term RNA silencing is used to describe all smRNA-mediated gene silencing pathways that are evolutionarily conserved in most eukaryotic organisms such as post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in nematodes, flies and mammals, and quelling in fungi.

1.2. Diverse RNA silencing pathways in plants

In plants, three RNA silencing pathways occur that seem to be involved in a variety of regulatory and immune functions (Fig. 1; Meins *et al.*, 2005). The first pathway regulates gene expression by microRNAs (miRNAs; Jones-Rhoades *et al.*, 2006) and *trans*-acting smRNAs (ta-smRNAs; Bartel, 2005; Vaucheret, 2005). Both predominantly direct cleavage of near-perfect complementary target mRNAs (Vaucheret *et al.*, 2004), but suppression of translation has also been observed in plants (Chen, 2004). The second, cytoplasmic pathway protects cells against the invasion of foreign nucleic acids, such as viruses and transgenes, through the action of ‘small interfering’ RNAs (siRNAs) that direct the cleavage of homologous RNAs (Lecellier and Voinnet, 2004). Viruses engineered to contain an endogenous sequence can trigger silencing of the endogene, which is termed ‘virus-induced gene silencing’ (VIGS; Baulcombe, 1999; Ruiz *et al.*, 1998). The third, nuclear pathway ensures genome integrity and defence against endogenous repeat DNAs, transposons and retroelements. It is related to transcriptional gene silencing (TGS), because it involves DNA methylation and/or chromatin modification by endogenous heterochromatic siRNAs (Chan *et al.*, 2004; Zilberman *et al.*, 2003; Zilberman *et al.*, 2004).

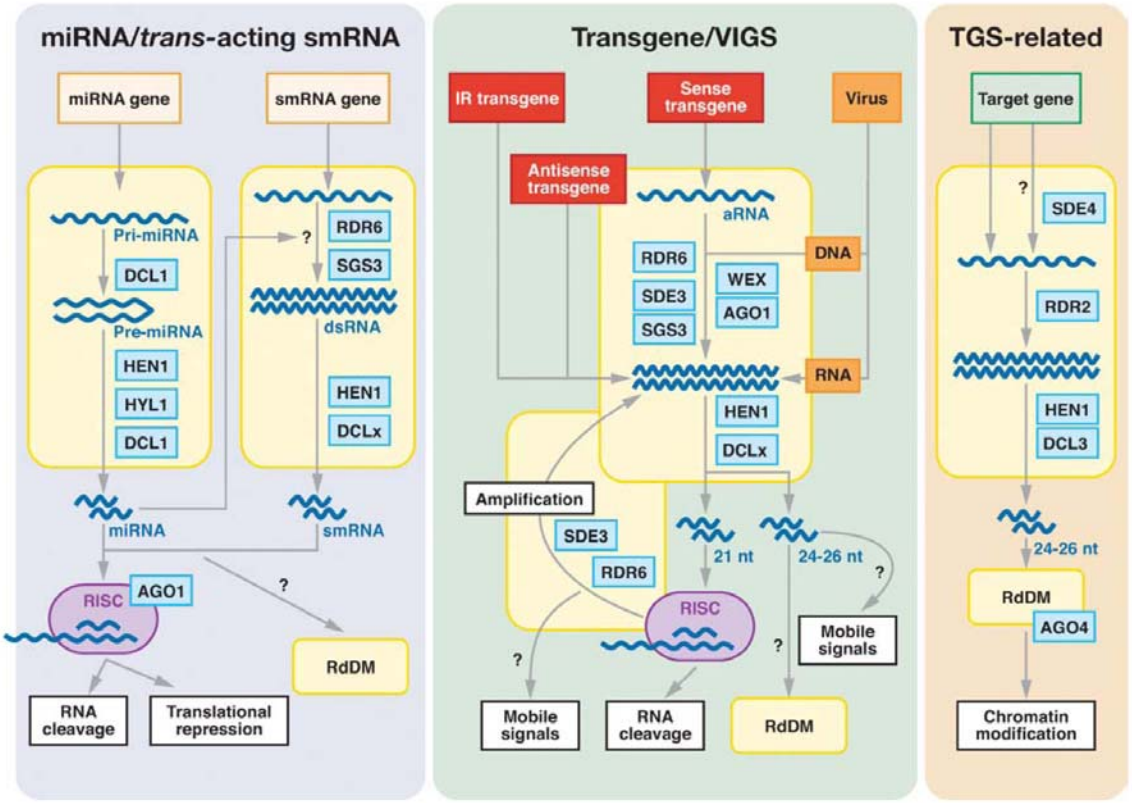

Figure 1. RNA silencing pathways in plants (*A. thaliana*; from Meins *et al.*, 2005). Functional modules are indicated in yellow and components identified by genetic analysis in pale blue. In the left pane, a model for miRNA and trans-acting smRNA biogenesis and action is given. In the middle pane, the pathway of transgene- and virus-induced silencing is depicted. In the right pane, the biogenesis and action of heterochromatic siRNAs is schematically summarized. DCLx refers to a postulated Dicer-like activity. See text for details and updates.

1.3. The mechanism of RNA silencing

The specificity determinants of RNA silencing are the smRNA molecules, which are generated through similar or partially overlapping pathways. They are derived from different sources of dsRNA (for review, see Kooter, 2005), which can be either the silencing trigger, e.g. miRNA precursors, RNA virus replication intermediates, or hairpin RNA (hpRNA) constructs, or an intermediate molecule that is formed by the action of an RNA-dependent RNA polymerase (RDR). The dsRNA is subsequently processed into smRNAs of 21-26 nt by an RNaseIII-like enzyme, known as Dicer-like or DCL. One strand from each siRNA duplex is stably incorporated into a silencing complex [for review, see Sontheimer *et al.*, 2005; RNA-induced silencing complex (RISC) or RNAi-induced transcriptional silencing (RITS) complex], and the other is degraded (Leuschner *et al.*, 2006). smRNAs in RISCs guide cleavage of complementary RNAs or translational suppression, whereas those in RITS complexes are associated with the establishment or maintenance of heterochromatin.

1.3.1. The miRNA and ta-smRNA pathway

The biogenesis and action of miRNAs and ta-smRNAs is schematically summarized in the left pane of Figure 1. Following transcription of the miRNA gene, most probably by RNA polymerase II (Xie *et al.*, 2005), the primary miRNA transcript (pri-miRNA) adopts a fold-back stem-loop structure that is processed to a miRNA precursor (pre-miRNA) and subsequently to a mature miRNA duplex by DCL1 (Park *et al.*, 2002; Reinhart *et al.*, 2002), perhaps with the aid of HYL1 (Han *et al.*, 2004; Vazquez *et al.*, 2004a) and other factors. The 3' sugars of the miRNA duplex are methylated by HEN1 (Yu *et al.*, 2005), to protect them from uridylation and associated destabilization (Li *et al.*, 2005). The miRNA is exported to the cytoplasm by the nuclear export receptor HASTY (HST; Park *et al.*, 2005), probably with the aid of additional factors. The mature, methylated miRNA duplex is incorporated into RISC that includes AGO1, and the so-called passenger strand of the duplex is degraded. Within the silencing complex, the miRNA is capable of targeting complementary RNAs for cleavage by AGO1 (Baumberger and Baulcombe, 2005; Vaucheret *et al.*, 2004), or for translational repression (Chen, 2004). The miRNA pathway regulates two of its own proteins, AGO1 (Vaucheret *et al.*, 2004) and DCL1 (Xie *et al.*, 2003). Recent evidence raised the possibility that miRNAs might direct DNA methylation (RdDM or RNA-directed DNA methylation; Bao *et al.*, 2004).

Recently, an additional role of miRNAs in the biogenesis of ta-smRNAs has been discovered. miRNA-directed cleavage of precursor ta-smRNA transcripts originating from ta-smRNA (*TAS*) genes seems to set the 21-nt phase for the production of the ta-smRNAs (Allen *et al.*, 2005). After RDR6- and SGS3-mediated conversion of one of the cleavage products into dsRNA, DCL4 processes the dsRNA into phased ta-smRNAs (Allen *et al.*, 2005; Gasciolli *et al.*, 2005; Yoshikawa *et al.*, 2005). Because their biogenesis resembles that of siRNAs, the ta-smRNAs are also designated ta-siRNAs, but they differ from all siRNAs described in plants in that they direct the cleavage of partially complementary target mRNAs that bear little resemblance to the *TAS* genes from which these siRNAs derive, hence the term *trans*-acting. Targeting occurs in an AGO1-dependent manner (*TAS1*- and *TAS2*-derived ta-siRNAs; Vazquez *et al.*, 2004b), but AGO7 also may play a role (*TAS3*-derived ta-siRNAs; Peragine *et al.*, 2004). Similar to the regulation of the miRNA pathway, ta-siRNAs also have the potential to regulate the accumulation of the *TAS* precursor RNA through a feedback loop (Vazquez *et al.*, 2004b).

1.3.2. The transgene- and virus-induced silencing pathway

The mechanism of transgene- and virus-induced silencing is presented in the middle pane of Figure 1. It consists of a branched pathway that converges on the production of dsRNA. Depending on the nature of the transgene and the virus, dsRNA is either formed directly by intra- or intermolecular basepairing [hpRNA constructs (Fig.1, IR transgenes) and antisense transgenes] or replication intermediates (RNA viruses), or through the action of RDR6 and other proteins (sense transgenes and DNA viruses). Accordingly, sense transgenes have been shown to require RDR6 for the induction of PTGS (Butaye *et al.*, 2004; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). The subsequent processing of the dsRNA into two classes of siRNAs (Hamilton *et al.*, 2002) is believed to depend on different DCL activities (Tang *et al.*, 2003). DCL3 produces the long siRNAs (24-26 nt) that may be involved in methylation (see Section 1.3.4.) and systemic silencing (Section see 2.3.1.). DCL4 produces the short siRNAs (21 nt; Dunoyer *et al.*, 2005) that can either guide RISC-mediated RNA degradation or spread to adjacent cells where they trigger RDR6-dependent systemic silencing (Himber *et al.*, 2003; Schwach *et al.*, 2005). DCL2 has been suggested to function in the antiviral defense pathway, although it is not the only DCL involved (Xie *et al.*, 2004). AGO1 is required for silencing induced by sense transgenes, but not by hpRNA constructs (Béclin *et al.*, 2002; Boutet *et al.*, 2003). The involvement of RNA amplification in transitive and systemic RNA silencing is discussed in more detail in Section 2 (Fig. 3).

1.3.3. The TGS-related pathway

In the right pane of Figure 1, the biogenesis and action of heterochromatic siRNAs is given. These siRNAs derive from intergenic regions, transposons, retro-elements or pseudogenes and a minority from protein-encoding genes (Llave *et al.*, 2002; Zilberman *et al.*, 2003). The plant-specific DNA-dependent RNA polymerase IVa (of which NRDP1a or SDE4 is the largest subunit) is believed to transcribe endogenous DNA repeats into RNA species that are made double-stranded by RDR2, followed by processing of the dsRNA into 24-26 nt long siRNAs by DCL3 (Herr *et al.*, 2005; Onodera *et al.*, 2005; Xie *et al.*, 2004). After methylation by HEN1, the heterochromatic siRNAs are incorporated into an AGO4-containing RITS complex mediating RdDM and chromatin modifications (Zilberman *et al.*, 2003; Zilberman *et al.*, 2004; see Section 1.3.4.).

Recently, a screen for mutants defective in hpRNA-directed promoter methylation and presumed TGS has identified a second RNA polymerase IVb. Whereas RNA polymerase IVa is involved in generating siRNAs, RNA polymerase IVb and an SNF2-like protein DRD1 are

required downstream of this step to induce RdDM by an unknown mechanism (Kanno *et al.*, 2005).

1.3.4. RNA-directed DNA methylation (RdDM)

All RNA silencing pathways appear to be associated with DNA methylation of the target genes. The first evidence that RNA molecules can direct DNA methylation came from experiments with viroid infection of tobacco plants. Viroids are plant pathogens that consist of single-stranded, circular rod-shaped RNAs that do not code for any proteins. Viroid cDNAs integrated in the tobacco genome became methylated only after autonomous viroid RNA-RNA replication had taken place (Wassenegger *et al.*, 1994). Detailed analysis demonstrated that cytosines in all sequence contexts were modified and that methylation was largely confined to the region of RNA-DNA sequence homology (Pélissier *et al.*, 1999). DNA regions as short as 30 bp could be targeted for methylation (Pélissier and Wassenegger, 2000).

De novo methylation at endogenous silent loci and transgenic sequences associated with transgene- and virus-induced gene silencing requires the synthesis and processing of dsRNA into 24-nt siRNAs, since loss of certain RNA silencing components necessary for siRNA accumulation can result in loss of cytosine methylation and decreased methylation of lysine 9 histone 3 (H3K9) at the targeted loci (Chan *et al.*, 2004; Hamilton *et al.*, 2002; Xie *et al.*, 2004; Zilberman *et al.*, 2004). The siRNAs would guide an RdDM-complex containing a plant-specific SWI2/SNF2-like chromatin-remodeling protein DRD1 (Defective in RdDM 1; Kanno *et al.*, 2004) to homologous sequences in the genome (Fig. 2). This complex would then recruit the major *de novo* DNA methyltransferases DRM1/DRM2 (Domains-rearranged methyltransferase) to establish *de novo* methylation in all sequence contexts (Cao *et al.*, 2003; Cao and Jacobsen, 2002a; Cao and Jacobsen, 2002b). The SWI2/SNF2-like ATPase DDM1 (decrease in DNA methylation; Jeddeloh *et al.*, 1999) might be targeted to the methylated DNA where it remodels the chromatine structure, exposing the DNA to the methyltransferase MET1 (Aufsatz *et al.*, 2004; Jones *et al.*, 2001) and other factors for the maintenance of CG methylation. The methyl binding protein MBD6 forms a complex with the histone deacetylase HDA6 (Zemach and Grafi, 2003), whose activity is also required to reinforce CpG methylation induced by RNA (Aufsatz *et al.*, 2002). The H3K9 methyltransferase SUVH2 [Su(var)3-9 Homolog 2] also depends on DDM1 and MET1 (Naumann *et al.*, 2005), potentially coupling histone methylation with chromatin remodeling and DNA methylation.

Maintenance of non-CG methylation depends on the H3K9 methyltransferase SUVH4/KYP (Kryptonite; Jackson *et al.*, 2002; Malagnac *et al.*, 2002). H3K9 methylation would make the

DNA accessible to the chromomethylase CMT3, which acts redundantly with DRM1/DRM2 to maintain non-CG methylation (Bartee *et al.*, 2001; Cao *et al.*, 2003; Cao and Jacobsen, 2002a; Lindroth *et al.*, 2001). Mutation of AGO4 blocks the accumulation of a subset of 25-nt siRNAs corresponding to endogenes or retroelements, which correlates with the loss of non-CG DNA methylation and H3K9 modification at the respective loci (Zilberman *et al.*, 2003; Zilberman *et al.*, 2004). The putative RITS-like complex containing an siRNA and AGO4 might target SUVH4 to methylate H3K9 (Naumann *et al.*, 2005; Wassenegger, 2005). However, mutation of AGO4 has much weaker effects on the maintenance of DNA methylation triggered by hairpin constructs: non-CG methylation is more extensively eliminated by crossing away the trigger than it is in the *ago4-1* mutant (Zilberman *et al.*, 2004). This implies the existence of an AGO4-independent pathway, possibly a redundant RNA silencing pathway that also directs methylation in response to these hairpin triggers.

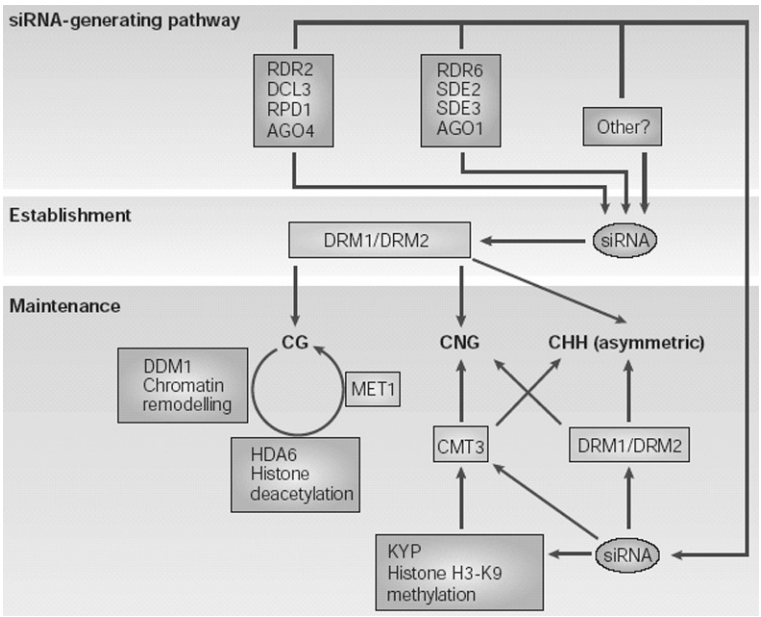

Figure 2. Establishment and maintenance of DNA methylation in *A. thaliana* (from Chan *et al.*, 2005). *De novo* methylation in all sequence contexts is entirely dependent on DRM1/DRM2, targeted by siRNAs generated by different RNA silencing pathways. The maintenance of CG methylation requires MET1, DDM1 and HDA6. Maintenance of non-CG methylation (CNG and CHH or CNN) involves CMT3 and DRM1/DRM2, which function with varying degrees of redundancy. CMT3 is directed by H3K9 methylation catalysed by KYP or by siRNAs. The latter can also guide DRM1/DRM2.

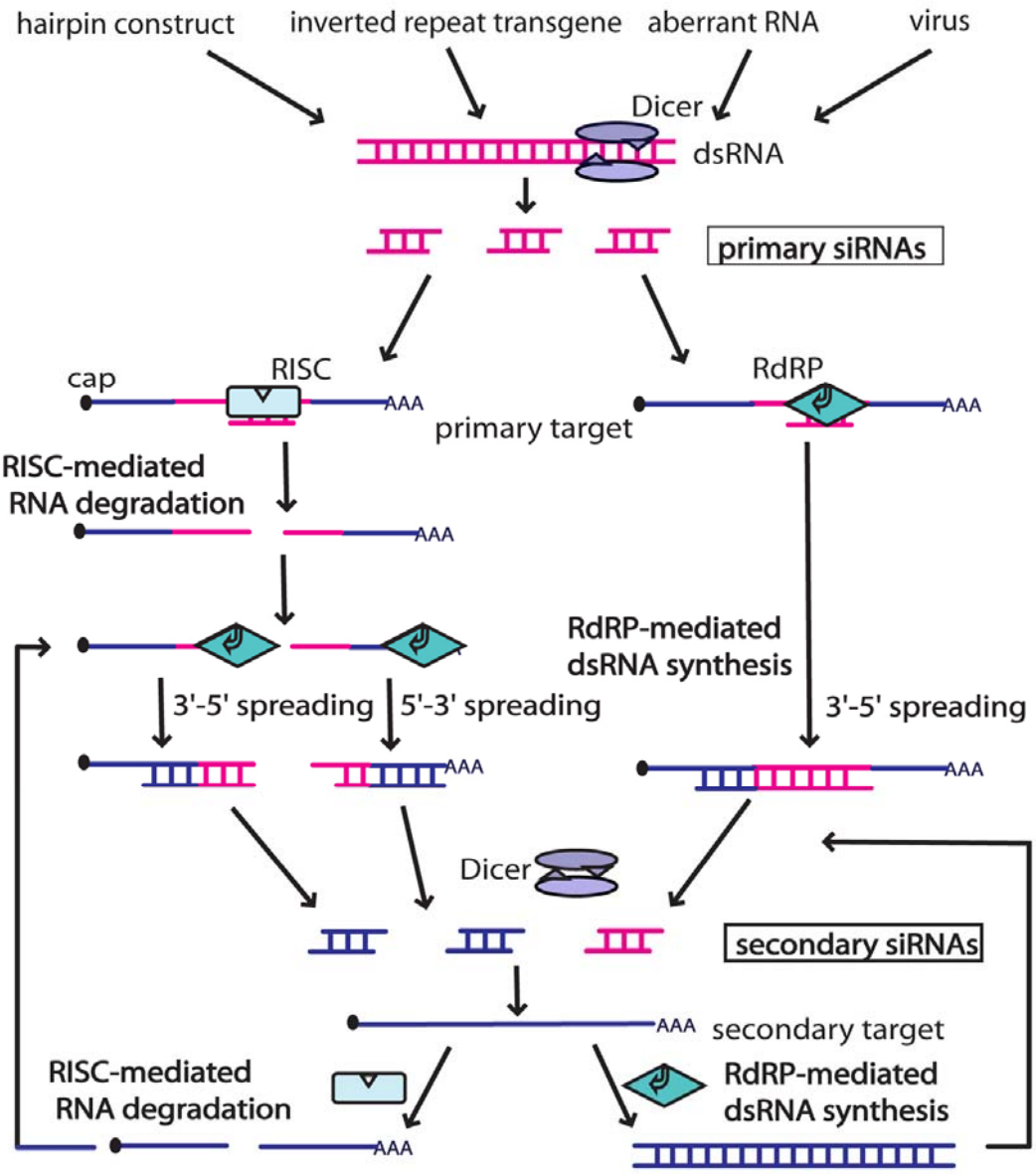

Figure 3. Scheme illustrating RNA amplification and transitive silencing (from Bleys *et al.*, 2006). The pink line in the primary target represents the sequence homology with the silencing inducer; the blue line indicates homology with the secondary target. The dsRNA trigger is cleaved by Dicer into primary siRNAs, which can subsequently follow two pathways. In the RISC-mediated degradation pathway they are incorporated into RISC, where they function as guides for cleavage of homologous RNAs. In the RDR/Dicer pathway target transcripts are indirectly degraded: first they serve as templates for RDR-mediated dsRNA synthesis, followed by cleavage by Dicer into secondary siRNAs. When the primary siRNAs are used as primers for the dsRNA synthesis, 3' to 5' spreading of RNA targeting can occur. In plants however, 5' to 3' spreading is also observed, which could be the result of unprimed dsRNA synthesis starting from the 3' end of an aberrant RISC-cleaved mRNA, either marked by an hybridizing siRNA or not. Unprimed synthesis could also account for 3' to 5' spreading when the capped 5' part of the RISC-cleaved mRNA is used as template. The produced secondary siRNAs can again follow the two pathways, resulting in silencing of a secondary target that has no sequence homology to the dsRNA inducer.

2. RNA amplification

2.1. RNA-dependent RNA polymerases

Early studies of RNAi in *C. elegans* have shown the remarkable potency of dsRNA: very small amounts of dsRNA are able to trigger silencing of a vast excess of target mRNA throughout the organism (Fire *et al.*, 1998; Sijen *et al.*, 2001). This striking observation suggested the need for an amplification step, without which siRNAs fail to reach sufficient concentrations to accomplish target mRNA degradation (Sijen *et al.*, 2001). Some degree of amplification is obtained by the conversion of the trigger dsRNA into many siRNAs, but this is not sufficient to bring about such continuous mRNA degradation. RDRs are very good candidate enzymes to be involved in the amplification of RNA silencing signals for efficient RNA silencing. Schiebel *et al.* (1993a, b) showed that the purified tomato RDR catalyzes the synthesis of dsRNA by using single-stranded RNA (ssRNA) as a template, both in a primed and unprimed fashion. Five years later they successfully cloned the gene (Schiebel *et al.*, 1998). Putative homologues of this gene are found in plants, fungi, and worms. Mutations in the *QDE-1* gene of *Neurospora crassa* (Cogoni and Macino, 1999), the *SGS2/SDE1/RDR6* gene of *Arabidopsis thaliana* (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000), the *EGO-1* and *RRF-1* genes of *C. elegans* (Sijen *et al.*, 2001; Smardon *et al.*, 2000), and the *RrpA* gene of *Dictyostelium discoideum* (Martens *et al.*, 2002) affect RNA silencing, demonstrating a clear genetic role for RDRs in the mechanism of post-transcriptional gene silencing. *Rdr6* gene silencing mutants even proved to be a valuable genetic background for transformation with overexpression constructs, increasing the frequency of highly expressing transformants from 20% to 100% in wild-type and *sgs2* backgrounds, respectively (Butaye *et al.*, 2004).

RDRs may operate at multiple levels in RNA silencing. They might be implicated in the generation of sufficient initial trigger dsRNA from speculative aberrant transgenic RNAs. In line with this hypothesis is the requirement of RDRs for PTGS triggered by sense transgenes (Butaye *et al.*, 2004; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000) and the observation that constructs that contain a sequence linked to a downstream IR of a heterologous 3'-untranslated region (UTR) are able to induce target gene silencing (Brummell *et al.*, 2003). Also, overexpression of the *N. crassa* RDR QDE-1 results in an increase in the production of siRNAs and a concomitant dramatic increase in efficiency of quelling induced by transgene tandem repeats, indicating that QDE-1 is a rate-limiting factor in quelling (Forrest *et al.*, 2004). In overexpressing strains, the number of transgene copies required to induce quelling is significantly reduced, suggesting that every transgenic/repetitive locus possesses the ability to produce a silencing signal, but that this signal is not always sufficient to induce silencing,

because the trigger dsRNA is expressed below a certain threshold. Silencing activation and maintenance in *N. crassa* appear to rely on the amount of both QDE-1 and transgenic copies that produce trigger RNA molecules to be converted to dsRNA by the RDR (Forrest *et al.*, 2004). Also consistent with a role of RDR in the production of sufficient trigger dsRNA is the observation that RDRs are no longer required when a preformed dsRNA is expressed from IR constructs in plants and fungi (Béclin *et al.*, 2002; Catalanotto *et al.*, 2004; Hammond and Keller, 2005; Schwach *et al.*, 2005), or when silencing is induced by viruses that encode their own RDR proteins (Dalmay *et al.*, 2000). On the other hand, in *C. elegans* (Sijen *et al.*, 2001), *Dictyostelium* (Martens *et al.*, 2002) and fission yeast (Schramke and Allshire, 2003) the RDR function is also required when the trigger dsRNA is delivered exogenously or expressed directly from hairpin constructs, suggesting that RDRs might participate in more than just the production of the dsRNA trigger, and that they are involved in amplification of the silencing signal leading to very efficient RNA silencing throughout the organism. The extent to which RDR action contributes to RNA silencing may depend on the organism or tissue, the specific induction pathway, or the target.

2.2. RNA amplification and transitive silencing

Amplification of the RNA silencing signal by an RDR could occur either by replicating the dsRNA trigger or by expanding the initial pool of siRNAs. The latter possibility is favored, because many studies support a model in which the primary siRNAs that are derived from the inducer dsRNA through Dicer activity serve as primers or are recognized as tags for synthesis of dsRNA by an RDR using the target transcript RNA as template. Subsequent cleavage of the produced dsRNA by Dicer results in the formation of new secondary siRNAs, in turn capable of targeting homologous target mRNAs for degradation. This RDR/Dicer pathway can also lead to the production of siRNAs corresponding to sequences located outside the region of homology between the silencing inducer and the primary target, thus resulting in the silencing of secondary targets that do not show any sequence homology to the initial silencing trigger. This kind of RNAi induced by secondary siRNAs spreading along the target gene, was designated transitive RNAi in *C. elegans* and occurs also in plants and fungi.

2.2.1. Transitive silencing in *Caenorhabditis elegans*

Analysis of RNA populations during RNAi in *C. elegans* demonstrated, in addition to the expected trigger-derived siRNAs, the existence of small RNAs that correspond to regions

upstream of the region targeted by the inducing dsRNA (Sijen *et al.*, 2001). The abundance of these secondary siRNAs appeared to decrease in function of the distance from the primary trigger, and to become negligible at distances greater than a few hundred base pairs. To test whether these secondary siRNAs are capable of targeting degradation of homologous mRNAs, a transitive RNAi assay was carried out, in which two populations of target RNA were present in the cell: a primary target with a segment of homology to the dsRNA trigger, and a secondary target with no homology to the trigger, but to the second segment of the primary target. In a first experiment, the primary target encoded a nuclear-targeted GFP-LACZ fusion protein, and the secondary target a mitochondrially targeted GFP. Injection of dsRNA segments from *lacZ* into animals carrying both transgenes resulted in the reduction of both nuclear GFP-LACZ and mitochondrial GFP. A trigger that was located just 3' to the *gfp-lacZ* junction was most potent in the transitive RNAi assay. When the same *lacZ* dsRNA trigger was used, but the order of segments in the primary target was reversed (LACZ-GFP), no transitive silencing could be obtained, consistent with the fact that no siRNAs downstream of the targeted region could be detected. In a second experiment, the primary and secondary targets are an *unc-22-gfp* transgene, and the endogenous *unc-22* gene, respectively. Injection of *gfp* dsRNA into animals expressing the *unc-22-gfp* transgene produced the twitching phenotype that is characteristic of loss of *unc-22* expression. To test whether transitive RNAi could proceed with endogenous genes as primary and secondary targets, animals heterozygous for a functional deletion allele of *unc-22* were injected with dsRNA corresponding to the deleted region, which led to transitive silencing of both the wild-type and the deletion allele.

Similar results were obtained by Alder *et al.* (2003), who could induce silencing of three different endogenous genes in *C. elegans* by transitive RNAi assays. Directionality for the primary mRNA target was observed: transitive RNAi targets mRNA sequences located 5' of the mRNA sequences homologous to the incoming dsRNA. The presence of the primary target mRNA is essential for the transitive effect, because it is presumed to function as a template for dsRNA synthesis. The most plausible explanation for the observed 3' to 5' directionality in *C. elegans* is that the siRNA functions as a primer to initiate elongation (Fig. 3; Lipardi *et al.*, 2001). Alternatively, differential stability of potential templates derived from a cleaved target mRNA could account for directionality. The 5' fragment may be more stable and therefore more frequently used as a template than the 3' fragment (Han and Grierson, 2002b). Finally, the machinery for RNA synthesis may be associated with RISC and positioned for unidirectional RNA synthesis by this complex (Alder *et al.*, 2003).

Interestingly, spreading could also be observed at the level of the dsRNA trigger (Alder *et al.*, 2003). A dsRNA hairpin consisting of a *gfp* stem and an *unc-22* loop is capable of inducing an Unc-22 phenotype, only when the hairpin trigger is continuously administered and a *gfp*

mRNA target is present. This observation implies that *unc-22* dsRNA is synthesized de novo via transitive RNAi from the hairpin construct itself by priming of cleaved *gfp* mRNA or *gfp* siRNAs with the hairpin dsRNA as template. In case of siRNA priming, the *gfp* transgene might be necessary to produce sufficient secondary siRNAs or structurally modified siRNAs, which could act as primers.

C. elegans has four RDR genes, *ego-1*, *rrf-1*, *rrf-2* and *rrf-3*. Smardon *et al.* (2000) demonstrated that *ego-1* is required for efficient RNAi in the adult germline. While the role of the other three RDRs in the transitive silencing process was investigated, *rrf-2* and *rrf-3* mutants were found sensitive to RNAi in all tissues (soma and germline) for both standard and transitive RNAi assays (Sijen *et al.*, 2001). Interestingly, the *rrf-3* deletion strain showed increased sensitivity to RNAi when compared to wild-type animals, perhaps because of competition with other RDRs (RRF-1 and EGO-1) for components or intermediates in the RNAi reaction (Simmer *et al.*, 2002). By contrast, in *rrf*-1 mutants, complete resistance against certain RNAi triggers in somatic tissue was observed, while interference was retained for genes expressed in the germline. Secondary siRNAs were not produced anymore, but the primary siRNAs corresponding to the original trigger RNA were still detected in *rrf-1* mutants. The lack of RNAi in the presence of primary siRNAs could have several reasons. First, the levels of primary siRNAs could be insufficient to trigger an efficient silencing response, perhaps because primary siRNAs are less efficiently incorporated into the RISC complex than the secondary siRNAs. Second, the initial siRNA-mRNA interaction may be relatively transient or unstable *in vivo* and polymerization by an RDR could be required for stabilization. Third, RRF-1 and other RDRs could have an additional function in the RNAi pathway, for example phosphorolyzing and breaking down the target RNA, or tagging it for destruction.

Additional support for involvement of RDRs in RNAi in *C. elegans* has been provided by Tijsterman *et al.* (2002) who showed that a broad range (22–40nt) of short antisense RNAs (asRNAs) can also efficiently trigger RNAi when injected in close proximity to the target mRNA. Because the asRNAs have less strict size requirements than double-stranded siRNAs, which are only functional when they are of 21-23nt in length (Elbashir *et al.*, 2001; Nykänen *et al.*, 2001), the asRNAs might enter the RDR/Dicer pathway, instead of the RISC-mediated mRNA degradation pathway. Like siRNAs, the asRNAs could act as primers for an RDR elongation reaction with the target RNA as template, leading to dsRNA molecules that can subsequently be a substrate for Dicer action (Tijsterman *et al.*, 2002). Consistently, modification of the 3' ends of the asRNAs severely reduced the silencing efficiency of the asRNAs (Tijsterman *et al.*, 2002), as observed in *Drosophila* embryo extracts, where double-stranded siRNAs were no longer incorporated into dsRNA when the 3' hydroxyl group was blocked by a phosphate group (Lipardi *et al.*, 2001). Because RDE-1 and RDE-4, which are required to initiate RNAi induced by

injection of dsRNA or by transgene-produced dsRNA (Grishok *et al.*, 2000) are dispensable for RNAi triggered by the asRNAs, the RDR-generated dsRNAs might be delivered to Dicer in a manner different from that of exogenous or transgenic dsRNA (Tabara *et al.*, 2002).

2.2.2. Transitive silencing in plants

Although the term transitive RNAi was first used in *C. elegans*, the phenomenon was initially observed in plants. Tomato transformants with extra copies of the tomato 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene (*aco1*) carrying an IR of the 5'-UTR, showed co-suppression of both the transgenic and endogenous *aco1* genes and, in addition, also of the endogenous *aco2* gene (Hamilton *et al.*, 1998). This latter gene only exhibits significant similarity to the *aco1* transgene in the coding region. This observation implies a spreading of the target region along the transgenic DNA or RNA from the IR in the 5'-UTR to the downstream coding sequence. This explanation was supported by the detection of 5'-UTR siRNAs and siRNAs corresponding to the region immediately downstream of the IR (Han and Grierson, 2002a). In transgenic *gfp*-expressing *Nicotiana benthamiana* plants, bombardment of 5' (GF) or 3' (P) fragments of *gfp* cDNA induced not only systemic silencing of the integrated *gfp* transgene, but also led to targeting of nonoverlapping *gfp* sequences that were independently expressed from a virus-based vector (PVX-P and PVX-GF, respectively; Voinnet *et al.*, 1998). These data demonstrate spreading of the PTGS target region in both 5' to 3' and 3’ to 5’ directions.

Also VIGS of transgenes is associated with target site spreading (Vaistij *et al.*, 2002). *N. benthamiana* plants expressing a chimeric *gfp-nos* transgene were first infected with three different viral vectors carrying the 5' or 3' half of the *gfp* coding region, or the 3'*nos* sequence (TRV:GF, TRV:P, or TRV:NOS). These vectors induced efficient post-transcriptional silencing of the *gfp-nos* transgene and the production of GF-, P-, and NOS-siRNAs in all three plants. These plants were challenge inoculated with an unrelated viral vector carrying the 3' half of the *gfp* coding region (PVX:P). This unrelated vector accumulated to low levels in all three plants, indicating that the P region of *gfp* was a target irrespective of whether the initiator of RNA silencing was GF, P, or NOS. Spreading occurred from the initiator region in both 3' (from GF to P) and 5' (from NOS to P) directions, extending at least through 332 nucleotides (P region), and depending on RDR function and on transcription of the *gfp-nos* transgene.

In contrast, VIGS of the endogenous genes phytoene desaturase (PDS) or ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) did not lead to resistance against the challenge inoculum (Vaistij *et al.*, 2002). Other reports demonstrated that hpRNA constructs were able to induce transitivity along transgenes, but not along endogenes (Himber *et al.*, 2003; Kościańska

et al., 2005; Miki *et al.*, 2005). Han and Grierson (2002b) showed that during co-suppression of an endogenous polygalacturonase gene and a 3' truncated homologous transgene, the siRNAs were most probably produced from the transgene, because no siRNAs from the 3' part of the endogenous transcripts were detected. All these studies suggest that endogenous target RNAs are incapable of entering the amplification pathway, because of possible quantitative or qualitative restrictions. Perhaps the endogenous transcripts are not numerous enough to serve as template (Tang *et al.*, 2003), or RDR6 is unable to recognize them as templates because the endogenous RNAs lack certain characteristics or are associated with proteins inhibiting RDR. On the contrary, Sanders *et al.* (2002) have shown that endogenous glucanase genes play an active role in the synthesis of siRNAs in tobacco protoplasts. During co-suppression of the endogenous *glb* gene and the *N. plumbaginifolia gn1* transgene (81% homology) small sense and antisense RNAs homologous to the endogenous *glb* gene were detected, indicating that the co-suppressed endogenous gene is involved in signal amplification and target selection.

The apparent discrepancy between the results of Sanders *et al.* (2002) and Vaistij *et al.* (2002) could be explained by the difference in silencing inducer: in comparison to viral RNAs, transgenes could more efficiently recruit endogenous targets into an active role in silencing, perhaps involving a nuclear phase that is absent during VIGS. In the experiments of Han and Grierson (2002b), it could be that the endogenous transcripts do serve as templates, after cleavage by RISC, and that the 5' half of the cleaved transcript, which is more abundant and perhaps more stable, is used preferentially as template. Some endogenous genes might also be more susceptible to transitivity than others, because of differences in transcript stability, abundancy, or secondary structure. Consistently, not all endogenous miRNA targets allow transitivity. The discovery of a miRNA complementary to a site that contains mutations in the *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) genes suggests that the mutant phenotype (McConnell and Barton, 1998; McConnell *et al.*, 2001) results from the persistence of *PHV* and *PHB* expression at developmental stages when these mRNAs are normally destroyed (Rhoades *et al.*, 2002). The dominant character of the *phv* and *phb* mutations observed in heterozygotes implies that miRNA-directed cleavage of the wild-type allele does not lead to the production of secondary siRNAs that could target the degradation of transcripts that originate from the mutated allele. This was supported by an *in vitro* experiment in wheat germ extracts (Tang *et al.*, 2003). In contrast, miRNAs were shown to trigger spreading of silencing along a GFP sensor construct, in which the GFP was transcriptionally fused to a miRNA 171 (miR171) target sequence (Parizotto *et al.*, 2004). The first report about miRNA-induced transitivity along endogenous sequences demonstrated that certain miRNAs set the correct cleavage register for ta-siRNA precursor processing (Allen *et al.*, 2005; see also 1.3.1.).

The differential susceptibility to transitivity may not only be the case for endogenous genes,

but also for transgenes, since transgenic RNA silencing systems that do not exhibit spreading of RNA targeting have been reported (Vogt *et al.*, 2004; Wang *et al.*, 2001). During silencing of a chimeric *gus*-*Sat* transcript induced by replicating satellite RNA only satellite-derived, but no *gus*-specific siRNAs could be detected (Wang *et al.*, 2001). In viroid-induced RNA silencing (VdIRS), PSTVd infection led to silencing of a chimeric *gfp* transgene containing part of the PSTVd sequence, but not of a simultaneously expressed *gfp* transgene without any PSTVd homology (Vogt *et al.*, 2004). This is striking since the *gfp* gene had been shown previously to be highly susceptible to transitive silencing in both the 5' and 3' directions (Vaistij *et al.*, 2002; Voinnet *et al.*, 1998). Because only PSTVd-specific siRNAs were detected, it was speculated that the inserted viroid sequence forms certain secondary structures that block RDR6-mediated dsRNA synthesis from the chimeric *gfp*-PSTVd transcripts (Vogt *et al.*, 2004).

Other reports on spreading of the target region in *N. benthamiana* plants undergoing VIGS, demonstrated a predominance of 5’ to 3’ spreading over 3’ to 5’ spreading (Braunstein *et al.*, 2002; Petersen and Albrechtsen, 2005), suggesting a link between the commonly observed preferential targeting of 3' regions of silenced transgenes and the RDR6-mediated spread of silencing (Braunstein *et al.*, 2002; English *et al.*, 1996; Hutvágner *et al.*, 2000; Sijen *et al.*, 1996; Van Houdt *et al.*, 1997). Small RNAs from the 3' region of *gus* could be observed after VIGS induction with PVX carrying a 5' *gus* sequence, but not vice versa (Braunstein *et al.*, 2002). This predominance could result from the disturbance or inhibition of RDR6 activity during unprimed elongation that started from the 3' end by certain features of the *gus* transcript in the 5' region, such as secondary structure. An alternative explanation might simply be the limited processivity of RDR6, allowing the polymerization of RNA only for a distance of a few hundred nucleotides from the 3' end.

Van Houdt *et al.* (2003) detected in a transitive silencing system in tobacco only *gus*-specific small RNAs derived from the 3' part of a *gus* transcript. In this system, a post-transcriptionally silenced IR transgene locus X (*npt*II-*3'chs* IR) can trigger silencing of a non-homologous transgene Z (*gus*-*3'nos*) when a stepwise homology is created through introduction of a chimeric primary target Y (*gus*-*3'chs*) with one region homologous to the silencing inducer X (*3'chs*) and a second upstream region homologous to the secondary target Z *(gus)*. Small *gus*-specific RNAs accumulate only upon silencing of the *gus* genes in the presence of locus X. These results fit the hypothesis of RDR-dependent synthesis of *gus* antisense RNA, primed by locus X-derived *3'chs*-specific small RNAs on a locus Y-derived transcript. Alternatively, mere association of the primary target transcript with an siRNA-protein complex, or partial RISC-mediated degradation could allow the transcript to be recognized by an RDR as template for dsRNA synthesis (Fig. 3; Van Houdt *et al.*, 2003). Primer-independent synthesis can explain the 5’ to 3’ spreading observed in plants; the tomato RDR is able to start polymerization

preferentially at the 3' terminus of the template in the absence of any primer (Schiebel *et al.*, 1993b). This mechanism could also account for the observed spreading of targeting in tobacco BY2 protoplasts: cotransfection with a *gfp* plasmid and siRNAs targeted to the 5' region of the *gfp* gene sequence demonstrate de novo synthesis of siRNAs corresponding to the 3' region of the *gfp* target (Vanitharani *et al.*, 2003).

2.2.3. Transitive silencing in fungi

An *in vivo* study in the protist *Dictyostelium discoideum* showed that target-dependent amplification of small RNAs by the RDR homologue RrpA is indispensable for hairpin RNA-induced silencing, which requires detectable amounts of small RNAs (Martens *et al.*, 2002). In the zygomycete *Mucor circinelloides*, silencing of the endogenous *carB* gene induced by an episomal 3' truncated transgene was associated with the production of small sense and antisense RNAs that also corresponded to sequences downstream of the transgenic inducer sequence (Nicolás *et al.*, 2003). The antisense RNAs were generated preferentially from the 3' region of the endogenous *carB* transcript, whereas antisense RNAs from the 5' region were hardly detectable, indicating that the majority of detected small RNAs are produced via primer-independent amplification by the putative RDR from the 3' end of the endogenous target mRNA, with low processivity. In contrast, in the filamentous fungus *Aspergillus nidulans* 5’ to 3’ transitive silencing was not detected (Hammond and Keller, 2005).

2.3. RNA amplification and systemic silencing

The observation that mRNAs are not only targets but also amplifiers of the initial silencing signal may not only explain the remarkable potency and observed transitivity of RNA silencing, but also the systemic propagation of RNA silencing throughout the organism. RNA silencing in plants and *C. elegans* is non-cell autonomous: it can be induced locally and then spread from the initiation site to more distant sites, and even persist a long time after the original source of silencing has been eliminated. In plants, two types of transmission occur: short-range cell-to-cell movement through plasmodesmata, and bidirectional long-distance movement via phloem. Systemic silencing not only requires a system to pass the silencing signal from cell to cell, but also a mechanism to perceive and amplify the incoming signal.

2.3.1. Systemic silencing in plants

The first hint for the systemic nature of RNA silencing came from studies in which non-clonal spatial patterns of co-suppression were observed: spontaneous induction of PTGS started on a localized area of a single leaf and then propagated through the whole plant as a gradual process (Boerjan *et al.*, 1994; Palauqui *et al.*, 1996). Direct evidence for a systemic silencing signal was obtained by grafting experiments (Palauqui *et al.*, 1997; Palauqui and Vaucheret, 1998; Voinnet *et al.*, 1998) and local delivery of exogenous DNA via *Agrobacterium tumefaciens* infiltration (Voinnet and Baulcombe, 1997; Voinnet *et al.*, 1998) or biolistics (Palauqui and Balzergue, 1999; Voinnet and Baulcombe, 1997). Grafting of the upper part (scion) of non-silenced plants onto the lower part (root stock) of silenced plants, has demonstrated that the silencing signal can be transmitted bidirectionally across a graft junction, but more efficient from root stocks to scions (Sonoda and Nishiguchi, 2000). The PTGS established in the grafted scions is maintained in the absence of the silenced root stock, but is not inherited to the next generation (Sonoda and Nishiguchi, 2000).

The signal produced in the silenced stock can pass through part of a stem of a wild-type plant in which no homologous target RNA is present (Voinnet *et al.*, 1998), but systemic co-suppression could only be observed when a transcriptionally active target transgene was present in the receiving cells, or when the endogenous target mRNA in the scion accumulated to high levels (Paddison *et al.*, 2002; Palauqui *et al.*, 1997; Palauqui and Vaucheret, 1998). These results suggest that the silencing signal can travel some distance through the phloem without any need for amplification, while in the receiving cells target-dependent amplification of the signal is required to obtain efficient silencing. Such amplification has been demonstrated during systemic PTGS induced by biolistically delivered siRNAs (Klahre *et al.*, 2002). New siRNAs representing parts of the target RNA that are outside the region of homology with the triggering siRNA could be detected in systemically silenced new leaves, indicating that siRNAs themselves or intermediates induced by siRNAs could comprise systemic silencing signals and that these signals can initiate an amplification cycle in the receiving cells. As mentioned above (section 2.2.2) Voinnet *et al.* (1998) also observed spreading of the target region during systemic silencing induced by bombardment of fragments of GFP, which can be explained by target-dependent amplification of the silencing signal. Another study demonstrated that the progression of locally induced silencing to systemic silencing is determined by the ability of the receiving cells to propagate the signal: only plants capable of triggering spontaneous silencing of a transgene and the homologous endogenous gene showed biolistic activation of systemic co-suppression (Palauqui and Balzergue, 1999). Also in grafting experiments only such plants were able to maintain the systemically induced co-suppression after grafting onto a wild-type

stock (Palauqui and Vaucheret, 1998).

Himber *et al.* (2003) studied the cell-to-cell movement of RNA silencing in more detail. *Agro*-infiltration of a *gfp* expressing *N. benthamiana* line consistently produced a fine red border over a constant number of cells (10-15 cells) in which *gfp* silencing was triggered by a signal originating from the infiltrated area, independent of the presence of homologous transcripts. Simultaneously induced silencing of a *gfp* transgene and an endogenous Rubisco small subunit (*RbcS*) gene by a chimeric phloem-restricted viral vector (PVX-GP:RbcS:P-Δ25) revealed two types of cell-to-cell movement: limited movement of silencing of the endogenous gene *RbcS*, which was restricted to 10-15 cells around the veins, and extensive movement of *gfp* silencing, which progressively invaded the entire lamina. This observation could be explained by a differential capacity of the target mRNAs to sustain transitivity (Vaistij *et al.*, 2002), because a similar experiment in *A. thaliana rdr6*- and *sde3* mutants showed that the extensive movement of *gfp* silencing depends on RDR6 and, to a lesser extent, on SDE3, an RNA helicase believed to be involved in RDR6-mediated dsRNA synthesis, whereas limited movement did not require any of those two proteins (Himber *et al.*, 2003). This hypothesis was confirmed by molecular analysis of *rdr6*- and *sde3* mutants: the production of secondary siRNAs (21 nt) through the action of RDR6 and SDE3 was necessary for extensive, but not for limited movement, which relied only on the presence of primary siRNAs (21 nt and 25 nt) that did not spread outside the region of homology with the hairpin trigger.

Experiments with the viral suppressor P1 (Voinnet *et al.*, 1999) showed that the 21 nt siRNAs were sufficient for short-range movement (Himber *et al.*, 2003), which was confirmed in a recent study analyzing mutant plants that lost the silencing movement phenotype (Dunoyer *et al.*, 2005). These observations and the fact that limited movement of *RbcS* gene silencing was associated with the production of mainly 21 nt siRNAs (Himber *et al.*, 2003) suggests that these molecules are the most probable candidates for the short-distance signal molecule, which is consistent with the fact that synthetic siRNAs can induce systemic silencing (Klahre *et al.*, 2002). Based on these results, a possible model for cell-to-cell movement of RNA silencing in plants has been proposed. Local initiation of silencing by *Agro*-infiltration, tissue-restricted VIGS or tissue-specific expression of a hairpin RNA would produce 21 nt and 25 nt primary siRNAs, but mainly the 21 nt siRNAs would move to adjacent cells, without any need for target RNAs. The outcome of this initial wave of limited movement depends on the presence of homologous transcripts capable of serving as templates for the synthesis of secondary 21 nt siRNAs through the action of RDR6 and SDE3. Like the primary siRNAs, the newly produced intermediates or derived secondary siRNAs could create a new wave of limited movement to adjacent cells, where they initiate the same RDR6/SDE3-mediated process. Such re-iteration of limited movement waves would eventually translate into extensive movement of RNA silencing

(Dunoyer *et al.*, 2005; Himber *et al.*, 2003). In accordance with this hypothesis, systemic disease resistance to plum pox virus, conferred by phloem-specific expression of hairpin dsRNA, is also associated with the RDR-mediated production of secondary siRNAs using the inoculated viral genome as a template (Pandolfini *et al.*, 2003). Recently, it has been proven that RDR6 is not required for the production or translocation of the systemic signal, but for the amplification of the signal in the receiving cells (Schwach *et al.*, 2005).

Hamilton *et al.* (2002) have shown that the 21 nt siRNAs were far more abundant than the 25 nt siRNAs in systemic tissues undergoing extensive cell-to-cell silencing movement, however the onset of systemic silencing in newly emerging leaves was associated with the 25 nt siRNAs. This suggests that there are separate signal molecules for cell-to-cell and long-distance transport. The 25 nt siRNAs (or a derivative/precursor molecule) could act as a phloem-specific silencing signal, whereas the 21 nt siRNAs are the short-distance signal molecules (Dunoyer *et al.*, 2005; Himber *et al.*, 2003). Several studies support the existence of separate mechanisms for cell-to-cell and phloem transport of RNA silencing. First, some silencing suppressors have contrasting effects on each transport process (Himber *et al.*, 2003). Second, systemic, but not local, spread of silencing can be blocked in the presence of low levels of cadmium (Ueki and Citovsky, 2001). Third, cell-to-cell RNA silencing movement can be independent of long-distance spread, which requires induction of RNA silencing in different cell types, including phloem cells (Ryabov *et al.*, 2004). However, other reports reveal no consistent correlation between the capacity for systemic silencing and the accumulation of any particular class of small RNA (García-Pérez *et al.*, 2004; Mallory *et al.*, 2003).

Another report that combined transitive and systemic silencing processes supported the suggestion that some kind of RNA amplification product is the systemic silencing signal (García-Pérez *et al.*, 2004). To investigate whether a primary target is capable of producing systemic silencing signals, a transitive silencing XYZ setup was used similar to that of Van Houdt *et al.* (2003), but instead of the three loci being present in the same plant genome, graftings were made: plants homozygous for the secondary target locus Z (*gus*-expressing ZZ scions) were grafted onto plants containing the silencer locus X and the primary target locus Y in different zygosity combinations (*gus*-silenced XXYY, XXY-, X-YY, and X-Y- stocks). Induction of systemic *gus*-silencing was dosage dependent: only a double dose of the silencer X and/or of the primary target Y in the stocks (XXYY, XXY- and X-YY) resulted in systemic silencing in the ZZ scions. The secondary target Z in the scion did not show any transcript homology with the silencing inducer X in the stock, indicating that a systemic silencing signal is not necessarily produced by the silencing trigger, but can also be generated from the primary target. Secondary siRNAs in XXY- and X-Y- stocks, which are capable and incapable of sending a systemic signal to the scion, respectively, have similar accumulation levels, suggesting that the mobile silencing

signal does not comprise the secondary siRNAs themselves, but rather some other RNA amplification product. Such product may be synthesized by an RDR using as template the transcripts derived from locus Y that are in some way marked, for instance by the 3'*chs* siRNAs derived from locus X. This marking may be a limiting factor to produce sufficient signal for systemic silencing, which might explain the observed dosage dependence: doubling the amount of 3'*chs* siRNAs and/or of *gus* mRNAs via homozygosity of locus X and/or locus Y, increases the probability of both molecules to find each other.

To activate systemic silencing not only amplification in the rootstock is required to produce enough systemic silencing signal, but also in the receiving scion to generate target-specific tertiary siRNAs that are able to establish silencing of the secondary target, as mentioned before (Section 2.2.2.; Palauqui and Vaucheret, 1998; Voinnet *et al.*, 1998). For the latter process the presence of the secondary target gene is needed, indicating that the target transcripts are involved in the perception and/or subsequent conversion of the systemic transitive signal into a pool of tertiary siRNAs by target-dependent amplification (García-Pérez *et al.*, 2004). Consistent with the hypothesis of RNA amplification products being the mobile silencing signal is the fact that transgenic tobacco with different silencing-inducing loci vary in their capacity to trigger systemic RNA silencing (Mallory *et al.*, 2003). A transgene IR-silenced line, which is able to induce systemic silencing, could efficiently enter the amplification pathway. It is hypothesized that there are sufficient target mRNAs that can serve as template in response to occasionally produced dsRNA from the transgenes integrated as an IR. On the contrary, two different amplicon-silenced lines fail to effectively transmit the systemic silencing signal; presumably no amplification can be initiated, because the target RNAs are efficiently degraded by rapidly assembled RISC complexes that are guided by siRNAs derived from the continuously produced dsRNA trigger (amplicon). A study in which RNA extracts prepared from *gfp*-silenced plants led to systemic RNAi after injection into *gfp*-expressing *C. elegans* revealed that an RNA of approximately 85 nt was most active in inducing silencing in the worm (Boutla *et al.*, 2002). This molecule, perhaps representing an RNA amplification product, could be responsible for the systemic spread in plants. Nonetheless, there are still many speculations about the nature of the systemic silencing signal(s) in plants.

Recently, a genetic screen for mutants with altered short-range silencing movement has identified three silencing movement deficient mutations (*smd1*, *smd2* and *smd3*) that probably affect factors involved in cell-to-cell trafficking of RNA silencing beyond the vasculature, and a single enhanced silencing movement (*esm*) mutant (Dunoyer *et al.*, 2005). Identification of the products of *SMD1*, *SMD2*, *SMD3* and *ESM* should contribute to a better understanding of the process of systemic silencing in plants.

2.3.2. Systemic silencing in *Caenorhabditis elegans*

In *C. elegans*, RNAi is also not restricted to those cells that are exposed to a dsRNA trigger, and even can be transmitted to progeny (Fire *et al.*, 1998). Systemic responses are observed by injecting dsRNA into any site of the nematodes (Fire *et al.*, 1998), by soaking them in a solution containing dsRNAs (Maeda *et al.*, 2001; Tabara *et al.*, 1998), or by feeding them with dsRNA-expressing bacteria (Timmons *et al.*, 2001; Timmons and Fire, 1998). Also tissue-specific expression of a hairpin *gfp* dsRNA trigger can lead to *gfp* silencing in other tissues, even in absence of a target gene in the dsRNA-expressing cells (Winston *et al.*, 2002), but this effect strongly depends upon the choice of promoter used to drive dsRNA expression (Timmons *et al.*, 2003). However, worms that did not exhibit comprehensive systemic RNAi phenotypes did show more robust systemic RNA silencing after treatment with exogenous, unrelated dsRNA. This demonstrates that dsRNA derived from the environment not only can trigger, but also influence RNA silencing mechanisms in nematodes (Timmons *et al.*, 2003). In this manner, systemic RNAi may be part of a general mechanism for sensing and responding to environmental pathogens.

Genetic screens for *C. elegans* mutants defective in the systemic spreading of RNAi have been performed by two independent groups. Winston *et al.* (2002) isolated three 'systemic RNA interference-deficient' (*sid)* mutants. The *sid-1* gene encodes a multispan transmembrane protein, enabling passive cellular uptake of dsRNA (Feinberg and Hunter, 2003). *Sid-1* mutants fail to exhibit systemic RNAi from transgene-derived and exogenously delivered dsRNAs, and show a reduced level of RNAi response in progeny of injected mutant animals (Winston *et al.*, 2002). Tijsterman *et al.* (2004) identified five 'RNAi spreading defective' (*rsd*) mutants, from which the *rsd-8* gene is the same as the *sid-1*gene. These mutants could be divided into two classes: *rsd-4* and *rsd-8* mutants that are completely defective in the cellular uptake of dsRNA, and *rsd-2*, *rsd-3*and *rsd-6* mutants that are not defective in the initial uptake of dsRNA from the gut into somatic tissues, but that are unable to further distribute this dsRNA to the germline.

As in plants, systemic RNAi in *C. elegans* also appears to involve transitive RNAi, but at the level of the trigger dsRNA. Introduction of a *gfp-unc-22* hairpin dsRNA with a *unc-22* single-stranded loop in strains expressing *gfp* only in the digestive tract, leads to efficient silencing of *unc-22*, which is exclusively expressed in the body wall muscles. This observation suggests that *unc-22* dsRNA, produced via transitive RNAi from the hairpin molecule, or some other RNA derivate, can move between cells (Alder *et al.*, 2003). In contrast, injection experiments with small asRNAs failed to induce systemic RNAi, although they are believed to enter the amplification pathway (Tijsterman *et al.*, 2002). More experiments are required to investigate the interplay between transitive and systemic RNAi in *C. elegans*.

2.3.3. Absence of transitive and systemic RNA silencing in *Drosophila* and mammals

RNAi in *Drosophila melanogaster* and mammals does not seem to involve the activity of an RDR, since their genomes do not code for members of the RDR family (Martinez *et al.*, 2002; Schwarz *et al.*, 2002). Furthermore, a variety of experiments argue against the need for RDR-mediated amplification in the RNAi pathway of *Drosophila* and mammals. First, cordycepin, an inhibitor of RNA synthesis, does not prevent dsRNA-induced targeting of endogenous mRNAs in mouse oocytes and early embryos (Stein *et al.*, 2003). Second, siRNA-mediated RNAi in human cells is transitory, with cells recovering from a single treatment in 4 to 6 days (Holen *et al.*, 2002; Kisielow *et al.*, 2002), suggesting that the original siRNAs are not amplified to sustain an RNAi response. Third, blocking the 3' hydroxyl termini of siRNAs does not influence their ability to efficiently trigger RNA silencing in *Drosophila* embryo lysate and in human cells (Chiu and Rana, 2002; Martinez *et al.*, 2002; Schwarz *et al.*, 2002). This observation is in contrast with the random degradative PCR model proposed upon the observation that siRNAs can act *in vitro* as primers for secondary dsRNA synthesis, for which the presence of both the 5' phosphate group and the 3' hydroxyl group is critical (Lipardi *et al.*, 2001). However, the RDR activity identified in *Drosophila* embryo extract could be unrelated to the RNAi mechanism. Fourth, dsRNA corresponding to an alternatively spliced exon selectively degrades specific alternatively spliced mRNA isoforms in cultured *Drosophila* cells, implicating the absence of transitive RNAi in *Drosophila* (Celotto and Graveley, 2002). Fifth, transitive effects directed to sequences downstream or upstream of the initial trigger region were not observed (Roignant *et al.*, 2003). Sixth, a recreation of the transitive RNAi assay of Sijen *et al.* (2001) in human cells, clearly showed a lack of transitive RNAi in these cells (Chi *et al.*, 2003). In the mosquito *Anopheles gambiae*, RNAi also does not show spreading outside the region of the target region (Hoa *et al.*, 2003). All these data imply that RDR-mediated transitive RNAi may represent an ancient phenomenon that was lost in higher animals.

In contrast to plants and nematodes, RNAi in *Drosophila* shows a strict cell autonomy. Cell-specific expression of a dsRNA trigger failed to induce spreading of RNAi throughout the fruitfly, perhaps because certain components are missing, such as transporters to export the signal from cells undergoing the RNAi process, and receptors for importing the signal in adjacent cells (Roignant *et al.*, 2003). In support for this hypothesis is the fact that the *Drosophila* genome does not seem to code for homologues of the *sid-1* gene of *C. elegans* encoding a transmembrane protein involved in dsRNA uptake in cells (Winston *et al.*, 2002). As demonstrated in plants, systemic RNAi might also require an amplification step to translate the mobile silencing signal into silencing-directing RNA molecules (siRNAs) in distant cells. The absence of detectable systemic RNAi in *Drosophila* might simply reflect the lack of detectable

transitive RNAi, which involves the de novo synthesis of secondary siRNAs. *Sid-1* and *rsd-3* homologues have been found in human and mouse (Kennedy *et al.*, 2004; Winston *et al.*, 2002), suggesting that RNAi in mammals could be systemic, but this still has to be demonstrated.

Why RDR-mediated amplification should be required for RNAi in some organisms, but not in others, is a question that remains difficult to answer. Transitive RNAi may have been lost in mammals because of the evolution of the interferon (IFN) response, which is induced by long dsRNA. This IFN response results in the general inhibition of translation by the protein kinase R (PKR) pathway, aspecific degradation of mRNAs by an induced RNase L, and finally apoptosis (Stark *et al.*, 1998), but the use of siRNAs can overcome this response. The presumed absence of an amplification mechanism in mammals may ensure that no aspecific responses are induced. A second possibility is the differential stability of primary siRNAs. In plants, nematodes, and fungi, siRNAs might be unstable and RDR-mediated formation of secondary siRNAs could increase the amount of siRNA-loaded RISC complexes either by amplification of the original pool of siRNAs, or by different features of the produced secondary siRNAs, leading to an increased stability or to more efficient incorporation into RISC. Supporting this hypothesis is the fact that *rrf1*-mutants show no detectable steady-state levels of siRNAs *in vivo*, whereas they retain the ability to produce a small population of siRNAs *in vitro* by RDR-independent cleavage of the original trigger (Sijen *et al.*, 2001). A recent paper describes the existence of an ‘siRNase’ (ERI-1), identified in a genetic screen for *C. elegans* mutants with enhanced sensitivity to dsRNA (Kennedy *et al.*, 2004). This ERI-1 protein and its human orthologue degrade siRNAs *in vitro*; presumably by affecting their 3’ overhangs, siRNAs become either non-functional and unable to enter RISC, or unstable and subsequently degraded by additional nucleases. Negative regulation of RNAi by ERI-1 may limit the duration, cell type specificity, or endogenous function of RNAi, and might operate more efficiently in *C. elegans* than in human cells. In *Drosophila* and mammals, siRNAs might be incorporated into RISC very rapidly and efficiently. *In vitro* experiments in human cell extracts show that RISC is formed during the first fifteen minutes of incubation and that the siRNAs are irreversibly associated with the protein components of RISC (Martinez *et al.*, 2002).

The fact that RNAi in *Drosophila* and mammals is not transitive or systemic, makes it a powerful tool for reverse genetics at the resolution of a single mRNA isoform and of a single cell type. RNAi triggered against one specific gene in a gene family or against one specific splicing variant of a gene will be limited to that one target. When induced in one specific tissue, RNAi will not spread to other tissues.

3. Applications of RNA silencing in plants

Since its discovery, RNA silencing has been widely implemented as a research tool for reverse genetics in plants and used for a number of potentially commercial applications in crop improvement by controlling unwanted traits or metabolic pathways (Senior, 1998). One of the first commercial products was a tomato with a longer shelf-life as a result of silencing of a gene required for softening of ripening fruit (Schuch *et al.*, 1989). Other plant varieties have been created that are resistant to viral infection (van den Elzen *et al.*, 1989), have an extended cut flower life (Savin *et al.*, 1995), or have a modified composition of storage compounds (Knutzon *et al.*, 1992). Transgenic poplars modified in lignine biosynthesis by silencing of the *CAD* gene require less chemicals to extract lignin from the pulp during the process of papermaking (Baucher *et al.*, 2003; Pilate *et al.*, 2002). PTGS is being usd to create decaffeinated coffee plants, because the industrial process to remove caffeine from coffee plants is expensive and results in a bad coffee taste (Ogita *et al.*, 2004; Ogita *et al.*, 2003).

Frequently used silencing systems are hpRNA-induced gene silencing (Helliwell and Waterhouse, 2005; Hilson *et al.*, 2004; Smith *et al.*, 2000), virus-induced gene silencing (VIGS; Lu *et al.*, 2003; Robertson, 2004) and the satellite virus-induced silencing system (SVISS; Gosselé *et al.*, 2002). For a recent review about the mechanism, advantages and disadvantages of these systems, I refer to Watson *et al.* (2005).

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Chapter 2:

Objectives

Objectives

This work contributes to a research field that aims at unraveling the process of RNA silencing, in particular of transitive silencing. In the first, application-oriented part of the thesis, we examined the potential of transitive silencing as a tool for functional genomics, and in the second, more fundamental part, we focused on factors that influence the efficiency of transitive silencing, such as the processivity of the key enzyme RDR6, the length of homology between primary and secondary target, and the presence of viroid sequences in the primary target.

Frequently used methods that exploit RNA silencing for gene discovery in plants are hairpin-induced or virus-induced gene silencing. Transitivity could be an alternative silencing technology, but in plants it has not been shown yet whether amplification products are able to induce silencing of endogenous targets. To address the first goal of this thesis, we developed a transitive XYZ system in *Nicotiana tabacum* (Chapter 3; Van Houdt *et al.*, 2003) and in *Arabidopsis thaliana* (Chapter 4). In both plant species we analyzed the ability of a sense transgene IR locus (X) to induce transitivity along a transgenic primary target (Y), resulting in silencing of a transgenic secondary target (Z) without homology to the silencing inducer. In tobacco, we also investigated the methylation status of the silencing inducer X and of the target sequences (Chapter 3). In *Arabidopsis*, we examined whether the *in trans*-silenced Y transcripts can produce a transitive silencing signal that is able to induce silencing of an endogenous gene target (Chapter 4).

The second goal of this work was to clarify some characteristics of transitive silencing. VIGS has been shown to spread over a distance of at least 1000 nt from the 5' end to the 3' end of the target mRNA, while 3' to 5' spreading can extend at least through 332 nt, with a possible limit of 600 nt (Petersen and Albrechtsen, 2005; Vaistij *et al.*, 2002). We examined the extent of 3' to 5' spreading induced by the IR locus X and looked at the influence of an increasing distance between primary and secondary target sequences in a primary target Y on the onset of transitive silencing of a transgenic target Z (Chapter 5). In the same study, we investigated the influence of the length of sequence homology between primary and secondary target on the frequency and efficiency of transitive silencing of an endogenous target. Finally, we examined whether inserting a viroid sequence into a primary target Y inhibited transitive silencing induced by IR locus X (Chapter 6), because results from the research group of M. Wassenegger recently demonstrated in tobacco that viroid-induced silencing did not induce spreading along a GFP-viroid fusion transgene (Vogt *et al.*, 2004).

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Chapter 3:

Transitive silencing induced by an invertedly repeated transgene locus in *Nicotiana tabacum*

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1. Spreading of RNA silencing and DNA methylation along RNA target sequences

Abstract

It is generally recognized that a silencing-inducing locus can efficiently reduce the expression of genes that give rise to transcripts partially homologous to those produced by the silencing-inducing locus (primary targets). Interestingly, also the expression of genes that produce transcripts without homology to the silencing-inducing locus (secondary targets) can be decreased dramatically via transitive RNA silencing. This phenomenon requires primary target RNAs that contain sequences homologous to secondary target RNAs. Sequences upstream from the region homologous to the silencing inducer in the primary target transcripts give rise to approximately 22-nt siRNAs, coinciding with the region homologous to the secondary target. The presence of these small RNAs corresponds with reduced expression of the secondary target whose transcripts are not homologous to the silencing inducer. The data suggest that in transgenic plants, targets of RNA silencing are involved in the expansion of the pool of functional siRNAs. Furthermore, methylation of target genes in sequences without homology to the initial silencing inducer indicates that not only RNA silencing can expand across target RNAs, but also that methylation can spread along target genes.

Introduction

RNA silencing is a conserved mechanism that occurs in various eukaryotic organisms and leads to targeted degradation of RNA sequences homologous to the trigger (reviewed in Matzke *et al.*, 2001; Sharp, 2001; Zamore, 2001; Hutvágner and Zamore, 2002). The potency of double-stranded RNA (dsRNA) in activating RNA silencing was first demonstrated in *Caenorhabditis elegans* and was designated RNA interference (RNAi) (Fire *et al.*, 1998). Also in plants post-transcriptional gene silencing (PTGS; for reviews, see Kooter *et al.*, 1999; Vaucheret *et al.*, 2001; Voinnet, 2001), which is mechanistically related to RNAi, can be efficiently elicited upon introduction of gene constructs that give rise to dsRNA (Hamilton *et al.*, 1998; Waterhouse *et al.*, 1998; Chuang and Meyerowitz, 2000; Smith *et al.*, 2000; Sijen *et al.*, 2001a; Wesley *et al.*, 2001; Stoutjesdijk *et al.*, 2002), or upon viral infection of plants that initiates production of dsRNA replication intermediates (Ruiz *et al.*, 1998; Baulcombe, 1999).

Sense and antisense small RNAs (approximately 20 to 25 nt), homologous with post-transcriptionally silenced sequences, accumulate specifically in various PTGS systems in plants (Hamilton and Baulcombe, 1999). Studies of RNAi in *Drosophila melanogaster* revealed that small RNAs result from symmetric processing of the dsRNA (Hammond *et al.*, 2000). Hamilton and Baulcombe (1999) first proposed that these small RNAs correspond to specificity determinants in PTGS and RNAi. These small RNAs have been shown to guide a nuclease complex to cleave single-stranded RNA with complementary sequences in Drosophila embryo lysates (Elbashir *et al.*, 2001a, 2001b). Therefore, the 21- to 23-nt RNAs are referred to as small interfering RNAs (siRNAs) or guide RNAs. Target mRNAs are cut in the center of the region recognized by the complementary guide RNAs (Elbashir *et al.*, 2001a), and mRNAs are cleaved only in the region corresponding to the dsRNA (Zamore *et al.*, 2000).

The amplification of the siRNA signal during RNAi in *C. elegans* has been investigated and, in addition to trigger-coincident siRNAs, populations of small antisense RNA have been detected that correspond to regions of the target RNA molecules located upstream of the initial trigger dsRNA, designated secondary siRNAs (Sijen *et al.*, 2001b). The abundance of secondary siRNAs seems to decrease in function of the distance from the region homologous to the primary trigger. Functionality has been demonstrated by means of a transitive RNAi assay (Sijen *et al.*, 2001b), in which two targets for silencing are provided. Similarly, plant viral vectors carrying part of a transgene elicit the production of transgene-specific, secondary siRNAs upon infection. As a consequence, these plants are protected against infection by an unrelated virus that carries another part of the transgene (Vaistij *et al.*, 2002). The RNA-dependent RNA polymerase 6 (RDR6) is required for the process of transitivity (Vaistij *et al.*, 2002; Himber *et al.*, 2003).

Plant RNA silencing is frequently accompanied by DNA methylation in symmetrical and non-symmetrical cytosines (Bender, 2001) in transcribed regions of the silenced genes (Ingelbrecht *et al.*, 1994; English *et al.*, 1996; Sijen *et al.*, 1996; Kovařík *et al.*, 2000; Van Houdt *et al.*, 2000a); although its role is still unclear. Sequence-specific methylation signals consisting of RNA-DNA associations are believed to be involved in methylating silenced genes (Wassenegger, 2000-2005). RNA-directed DNA methylation (RdDM) was first discovered in tobacco plants that contained multimeric genome-integrated copies of a viroid cDNA (Wassenegger *et al.*, 1994). In these plants, specific *de novo* methylation that is restricted to the cDNA region was detected whenever viroids replicated autonomously (Pélissier *et al.*, 1999). Further, viroid-infected plants accumulate small RNAs identical in size to those found in plants exhibiting PTGS of transgenes (Papaefthimiou *et al.*, 2001). These results suggest that viroid-related RNAs induce methylation of the homologous cDNA copies. However, it remains controversial whether silencing-triggering dsRNA molecules, small guide RNAs, or intermediate RNA products are the signals for methylation of homologous DNA. PTGS induced by viral RNA that carries a short region homologous to the transgene leads to spreading of methylation throughout the transcribed region of the transgene (Jones *et al.*, 1999; Thomas *et al.*, 2001; Vaistij *et al.*, 2002). Direct interaction between the input recombinant virus and the homologous transgene might lead to RdDM, and the progressive degradation of target mRNA (Zamore *et al.*, 2000) could release more fragments, which additively direct methylation throughout the transcribed region of the transgene (Thomas *et al.*, 2001). Alternatively, viral dsRNAs, synthesized by a putative plant RDR (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000) or its derived siRNAs, could mediate RdDM (Vaistij *et al.*, 2002).

Here, we address the question whether the *in trans*-silencing capacity of a silencing-inducing transgene locus can be transmitted to a target RNA, subsequently able to silence secondary targets *in trans*. Therefore, we tested whether a post-transcriptionally silenced transgene locus can silence *in trans* a secondary target, which only produces non-homologous transcripts. To this end, we created a stepwise homology between the silencing inducer and the secondary target by producing a transcript from a primary target with one region homologous to the silencing inducer and another region homologous to the secondary target mRNA. We investigated the production of small, approximately 22-nt long RNAs, corresponding to target mRNAs and analyzed the methylation status of sequences silenced *in trans* in the region non-homologous to the silencing inducer.

Results

A post-transcriptionally silenced inverted repeat transgene locus can trigger silencing of a reporter gene producing non-homologous transcripts

To determine whether the RNA silencing activity of a silencing-inducing RNA can be transmitted to another RNA sequence by fusing this sequence to part of the silencing-inducing sequence in a single transcript, we studied transgenic tobacco plants with different combinations of three transgene loci (locus X, Y and Z). Locus X (Fig. 1) harbors an IR about the right T-DNA border of T-DNA GVCHS287, carrying a neomycin phosphotransferase II (*npt*II) gene under control of the cauliflower mosaic virus 35S promoter (CaMV P35S) and the 3' signaling sequences of the chalcone synthase gene (3'*chs*) of snapdragon (*Antirrhinum majus*) (Van Houdt *et al.*, 2000a, 2000b; Fig. 1). The two convergently transcribed *npt*II genes in locus X produce only very low amounts of the NPTII protein compared to those produced by a single-copy *npt*II transgene. The invertedly repeated *npt*II transgenes in locus X had been shown to be post-transcriptionally silenced and methylated in the 3' half of the genes (Van Houdt *et al.*, 2000a). Locus Y (Fig. 1) contains a single copy of the T-DNA GUSchsS and harbors a chimeric β-glucuronidase (*gus*) gene under control of P35S and 3'*chs* with an artificial intron in the 5' region of the coding sequence. In tobacco plants hemizygous for locus Y, the *gus* expression levels are normal (Table 1). Locus Z has two or more copies of T-DNA XD610 with a *gus* gene under control of P35S and the 3'-untranslated region (UTR) of the nopaline synthase gene (3'*nos*). In locus Z (Fig. 1) the *gus* expression is stable (Table 1). Here, the *in trans*-silencing effects between these three transgene loci X, Y and Z were studied in hybrid transgenic tobacco plants with any possible combination of these three loci under hemizygous condition, obtained by crossing the appropriate parental plants (see "Materials and Methods"). *In trans*-silencing effects were revealed by a reduced GUS activity of particular loci in certain combinations. The results of the GUS activity measurements in protein extracts of different types of hybrid plants are summarized in Table 1.

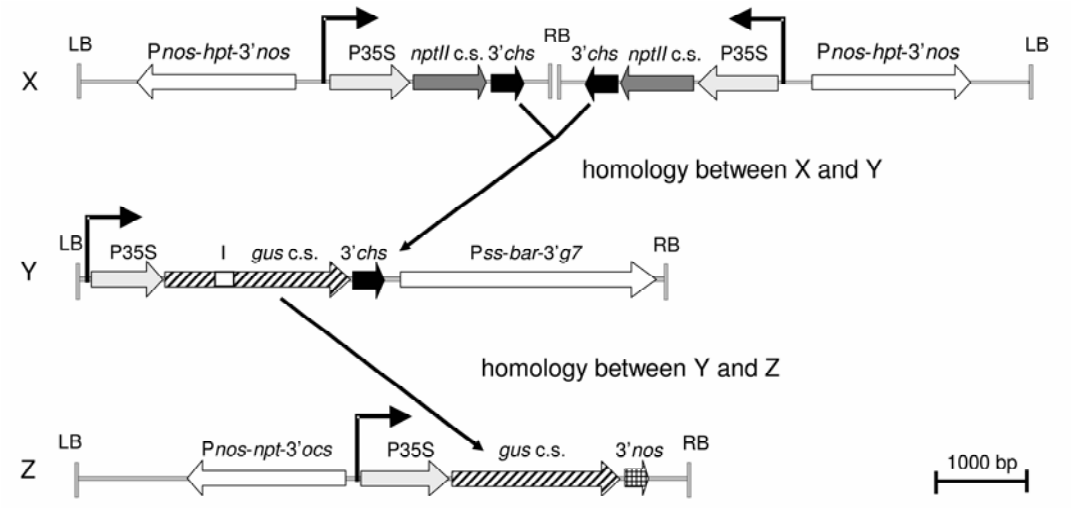

Figure 1. Schematic outline of the T-DNA constructs (drawn to scale), present in silenced locus X, recombinant gene Y, and target gene Z (T-DNAs of pGVCHS287, pGUSchsS, and pXD610, respectively), and of the transcript homology between X, Y, and Z. The structure of locus X and Y is indicated; locus Z contains two or more copies of the XD610 T-DNA. 3'*chs*, 3'-UTR of the chalcone synthase gene of snapdragon (*Antirrhinum majus*); 3'g7, 3'-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7; 3'*nos*, 3'-UTR of the nopaline synthase gene; 3'*ocs*, 3'-UTR of the octopine synthase gene; *bar*, bialaphos acetyltransferase-coding sequence conferring phosphinothricin resistance; *gus* c.s., β-glucuronidase-coding sequence; *hpt*, hygromycin phosphotransferase-coding sequence; I, artificial intron; LB, left T-DNA border; *npt*II c.s., neomycin phosphotransferase II-coding sequence; P35S, cauliflower mosaic virus 35S promoter; P*nos*, nopaline synthase promoter; P*ss*, promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase; RB, right T-DNA border.

Table 1. GUS activity determination in protein extracts of leaf tissue harvested from tobacco plants containing different combinations of loci X, Y and Z (Fig. 1).

Genotype	GUS activity [1]	No. of plants analyzed [2]
X	Below detection	1
Y	368 ± 165	9
Z	126 ± 60	10
XY	2 ± 1	4
XZ	139 ± 35	9
YZ	477 ± 101	10
XYZ [3]	4 ± 3	22

[1] Mean ± standard deviation, in Units GUS mg^{-1} total soluble protein.

[2] The axenically grown plants were analyzed 4 weeks after sowing on Murashige and Skoog medium supplemented with 1% sucrose in Falcon Petri dishes (Becton Dickinson, Bedford, MA).

[3] XYZ plants were grown in the presence of phosphinothricin (10 μg/ml) and hygromycin (25 μg/ml);

under these conditions, both XYZ plants and YZ plants containing the pNE T-DNA (see "Materials and Methods") were able to develop. A polymerase chain reaction with X-specific primers was performed to screen for the presence of X.

Previously, the silenced *npt*II genes in locus X have been demonstrated to silence *in trans* homologous *npt*II transgenes (Van Houdt *et al.*, 2000a) and transiently expressed genes with partial transcript homology to locus X-derived *npt*II transcripts (Van Houdt *et al.*, 2000b). We observed that also the stably expressed *gus* gene in locus Y, with partial transcript homology to the *npt*II transcripts of the silencing-inducing locus X (Fig. 1), was silenced efficiently *in trans* (compare XY with Y; Table 1). The homology between the transcripts of X and Y was mainly situated in the 3'-UTR (206 nt), but also the 5'-UTR had a small region of homology (29 nt). This relatively short region of homology between locus X-derived *npt*II and locus Y-derived *gus* transcripts was sufficient to degrade very efficiently Y-derived transcripts. To assess the stability of *in trans*-silencing of Y in XY hybrids, 4-week-old phosphinothricin-resistant progeny plants of a self-fertilized XY hybrid were analyzed. Loss of locus X in the progeny plants, as revealed by kanamycin sensitivity, correlated with reactivation of *gus* expression in locus Y in the expected 1:4 ratio, indicating that the *in trans*-silenced phenotype is not transmitted to the next generation when the silencing-inducing locus X is absent. In contrast to the low GUS activity detected in XY hybrids, the GUS activity in XZ hybrids was normal and similar to that in Z plants (Table 1). This observation allows us to conclude that the *npt*II genes of locus X could trigger neither transcriptional silencing of the *gus* genes in locus Z, although also driven by the CaMV P35S promoter, nor post-transcriptional silencing of the *gus* genes in locus Z, which was expected because both loci produce transcripts without significant homology (Fig. 1). These data, in addition to results of run-on transcription analyses of locus X-containing plants (Depicker *et al.*, 1996; unpublished results), support that the *in trans*-silencing effects in XY hybrid tobacco plants are not triggered by P35S homology. When Y and Z loci were combined in so-called YZ hybrids, the tobacco cells produced two types of *gus* transcripts with a 1809 nt homologous region in the *gus*-coding sequence. Both types of *gus* genes, however, remained normally expressed as reflected in the high GUS activity in YZ hybrids, and displayed a dosage effect, as expected for normally expressed genes (compare YZ with Y and Z; Table 1). Thus, the RNA silencing mechanism was not activated in YZ hybrid tobacco plants. Therefore, it is interesting to observe that upon creation of a stepwise homology between X and Z by the presence of locus Y, the *gus* expression in locus Z together with that of locus Y, was reduced in XYZ hybrid tobacco plants (compare XYZ with YZ; Table 1). As shown schematically in Figure 2, silencing of the target could be triggered by a stepwise homology that was created between a silenced locus (X) and a non-homologous target gene (Z) by introducing a chimeric recombinant gene with one region homologous to the silenced locus (X) and another homologous to the target (Y).

We refer to this as a case of transitive silencing in plants in which the silencing inducer (X), as well as the primary and secondary targets (Y and Z) are all nuclearly expressed transgenes. The results imply that the silencing capacity of locus X is transferred to Y sequences upstream of the homology between X and Y.

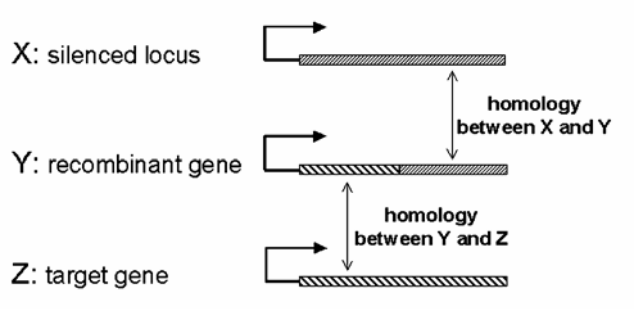

Figure 2. Schematic outline of homology between silenced locus X, recombinant gene Y, and target gene Z, resulting in silencing. The angular arrows and hatched boxes represent promoters and transcribed sequences, respectively.

In trans-*silenced loci give rise to approximately 22-nt siRNAs*

To understand the observation that locus Z is silenced only in XYZ hybrids through a transitive silencing effect and to confirm that silencing capacities of silencing-inducing loci can expand to target genes, we assessed the accumulation of sequence-specific siRNAs. First, we determined whether siRNAs specific for the silencing-inducing locus could be detected. RNA gel blots using hydrolyzed *npt*II transcripts as a probe (Fig. 3A) revealed locus X-specific *npt*II 22-nt siRNAs in the low-molecular weight RNA fraction of plants hemizygous for locus X and of XYZ hybrid tobacco plants. Wild-type SR1 plants (Fig. 3A, lane 1), hybrid lines without the *npt*II transgene (Fig. 3A, YZ in lanes 6 to 8) as well as a transgenic line containing a normally expressed *npt*II transgene (Helo2; Van Houdt *et al.*, 2000a; data not shown) did not accumulate small *npt*II RNAs. We examined the ability of target loci to give rise to target-specific siRNAs. Therefore, we used hydrolyzed *gus* transcripts as a probe to detect siRNAs originating from the *gus* sequences that do not occur in the silencing-inducing locus X. Only XYZ hybrids (Fig. 3B, XYZ in lanes 3 to 5), which show transitive silencing, but not YZ hybrids, X-hemizygous plants, nor wild-type SR1 (Fig. 3B, lanes 6 to 8, 2, and 1, respectively), accumulated small *gus* RNAs, although an identical set of *gus* genes was present in the XYZ and YZ hybrid lines. To determine which region of the *gus* transcripts is mainly giving rise to the detected small *gus* RNAs (Fig. 3B), we performed two RNA gel blots with identical RNA samples hybridized with different nonoverlapping partial *gus* transcripts as probes (Fig. 3C); one (5'*gus*) spanned the most 5' 800 bp of the *gus*-coding sequence, the other (3'*gus*) the most 3' 800 bp. A clear signal

was obtained with the 3'*gus* probe for XY and XYZ samples (Fig. 3C, upper panel, lanes 2 and 3), but no signal was detected with the 5'*gus* probe (Fig. 3C, lower panel, lanes 2 and 3), although the controls (Fig. 3C, c in both pannels) gave signals of comparable intensities with both probes, assuring similar probe quality and quantity. We conclude that small *gus* RNAs accumulate only upon silencing of *gus* genes in the presence of locus X and are mainly derived from the 3' part of the *gus* transcripts. Further, a weak signal corresponding to 3'*chs*-specific small RNAs was detected in samples of XY and XYZ hybrids that showed *gus* silencing, whereas these molecules were not detected in samples of YZ hybrids with normal *gus* expression (Fig. 3D, lanes 2 and 3, 5 and 6, and 7 and 8, respectively). Therefore, we suggest that small RNAs corresponding to the 3'*chs*-UTR region may direct the formation of small RNAs of more upstream-located sequences.

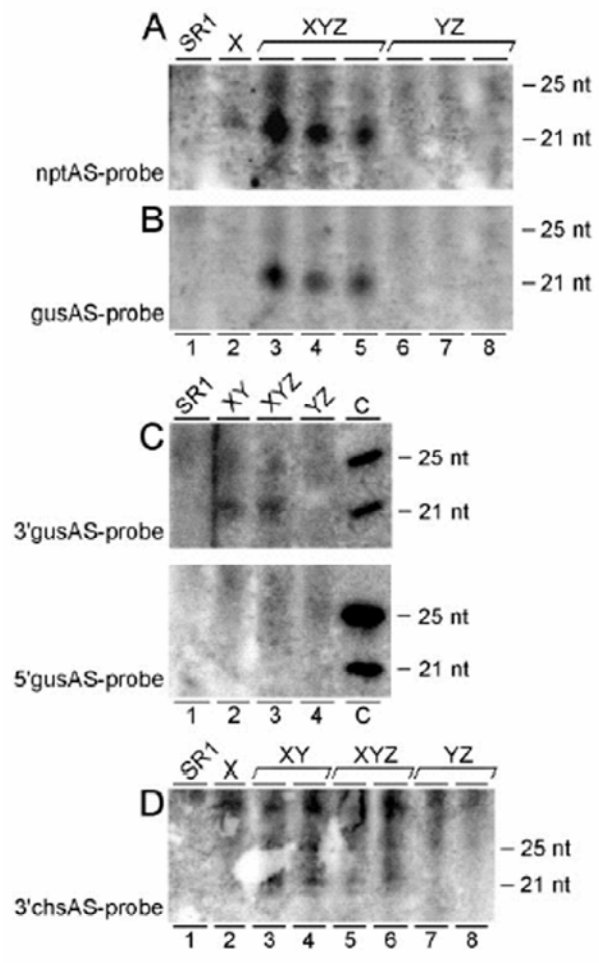

Figure 3. Detection of small RNAs. Low-molecular weight RNA fractions were isolated from leaf tissue of mature non-flowering tobacco plants, separated on polyacrylamide gels, blotted onto Hybond N^+ membranes, and hybridized with hydrolized antisense-specific riboprobes: *npt*II (full-length *npt*II-coding sequence; A), *gus* (full-length *gus*-coding sequence; B), 5'*gus* (most 5' 800 bp of the *gus*-coding

sequence; C, upper panel), 3'*gus* (most 3' 800 bp of the *gus*-coding sequence; C, lower panel), and 3'*chs* (3'*chs*-UTR sequence; D). DNA oligomers were used as size controls (size indicated in nucleotides). Each numbered lane contains the low-molecular weight RNA fraction of another tobacco plant of the genotype indicated on top. For the wild-type tobacco SR1 and the normal *gus*-expressing hybrid plants YZ, no specific signal could be detected with either of the probes. Locus X-containing plants (X and XYZ) gave rise to small *npt*II-specific RNAs of approximately 22 nt (A). The XYZ plants showing *gus* silencing accumulated approximately 22-nt small *gus*-specific RNAs (B). In *gus*-silenced XY and XYZ plants, these siRNAs corresponding to the 5' *gus* probe do not accumulate to detectable levels (C, upper panel), whereas those corresponding to the 3' *gus* probe give a detectable signal upon identical exposure time (C, lower panel). The lanes indicated by 'c' (controls) contain 3.3 picomols 21-nt long DNA and 2.5 picomols 25-nt long DNA with a GC content of 72% and 71.4%, respectively, and are shown for comparison of probe quality and quantity in upper and lower panel; the control DNA oligonucleotides in the upper and lower panels are 100% complementary to a stretch in the 5'*gus* probe and 3'*gus* probe, respectively (C). Approximately 22-nt small 3'*chs*-specific RNAs were, although expected for all X-containing samples, only detected in XY and XYZ samples (D).

DNA sequences that are non-homologous to the silencing-inducing locus X become methylated upon inactivation

The silencing-inducing locus X contains two invertedly repeated, convergently transcribed *npt*II transgenes that are extensively methylated in the center of the repeat (Van Houdt *et al.*, 2000a). In addition, a completely homologous *npt*II transgene becomes methylated upon *in trans*-silencing by locus X (Van Houdt *et al.*, 2000a). Does *in trans*-methylation rely on sequence homology with the (initial) silencing-inducing locus or can target sequences silenced *in trans* be involved in producing the methylation signal? To address these questions, we examined the methylation status of several cytosines located in the *gus*-coding sequences in the genomic DNA of non-silenced hemizygous Y, Z, XZ and YZ hybrids and silenced heterogenous XY (progeny obtained after self-fertilisation of hemizygous XY hybrids) and silenced hemizygous XYZ plants via DNA gel blot analysis with the methylation-sensitive restriction enzyme *Hpa*II. Figure 4A shows the *gus*-coding sequences in the T-DNAs of loci Y and Z, the location of methylation-insensitive *Hpa*I and *Sca*I restriction sites, used to delimit the analyzed region of the T-DNA, and of the methylation-sensitive *Hpa*II restriction sites, and the sizes of the digestion products of non-methylated *gus* transgenes. *Hpa*II recognizes the sequence CCGG and will not cut this sequence in case at least one of the cytosines is methylated. Upon complete digestion with *Hpa*II of the *gus* transgenes in locus Y and Z, only relatively small hybridizing DNA fragments will be detected, with the largest one being 427 bp (Fig. 4A). This result was obtained with different samples of Y (Fig. 4B; lane 1), Z (lane 2) and XZ (lanes 3 and 4) genomic DNA analyzed in a triple digest *Sca*I/*Hpa*I/*Hpa*II. Two YZ samples (lanes 5 and 6) showed a faint band of approximately 700 bp [Fig. 4B; indicated with (a)], but the *gus* probe clearly revealed more intense bands with a higher molecular weight in the XY (lanes 7 and 8) and XYZ (lanes 9

to 11) samples compared to the YZ samples, indicating strongly enhanced cytosine methylation upon silencing of the *gus* genes. The YZ hybrids originated from the cross between hemizygous XY plants and homozygous Z plants, by selection for the presence of locus Y and screening for the absence of locus X. Thus, it seems that the imprinting in CG context is to some extent maintained in the absence of the silencing-inducing locus 1. The size of the hybridizing bands with higher molecular weight (approximately 0.7 kb, 1.0 kb, 1.1 kb, 1.2 kb and 1.4 kb; Fig. 4B) suggests that at least three cytosines located in the 3' half of the *gus*-transcribed sequences were methylated in XYZ tissues. The detection of bands of about 1.1 kb and 1.2 kb [Fig. 4B; indicated with (b)] suggests that not only locus Y, but also locus Z became methylated upon inactivation. Also, it is clear that the intensity of methylation in different XYZ plants is variable. One XY sample (Fig. 4B; lane 8) appears to show methylation of all restriction sites except the most upstream CCGG site, because no band of approximately 0.4 kb and only a faint band of about 0.25 kb were detected, but instead intense bands of approximately 1.5 kb, 1.8 kb and 2.3 kb were visible. Because this XY plant is a descendant of a self-fertilized hemizygous XY plant, it might contain one or both loci X and Y in homozygous condition, which could explain the strong methylation spreading into the 5' half of the *gus*-transcribed sequence in locus Y. Alternatively, passage through meiosis could account for this observation. Collectively, these data suggest that primary and secondary target genes become methylated upon *in trans*-silencing in a region without homology to the silencing-eliciting transgenes and that corresponds to the region giving rise to small *gus* RNAs.

To confirm these results and to analyze the distribution and density of methylated cytosines in both loci Y and Z in more detail, we performed bisulfite genomic sequencing (Frommer *et al.*, 1992). This method allows precise analysis of the methylation status of all cytosines in the region of interest. Bisulphite treatment of genomic DNA leads to the deamination of non-methylated cytosines into uracil, while the methylated cytosines remain non-reactive. After PCR-amplification and sequencing, non-methylated cytosines are converted to thymines in the obtained sequence, while methylated cytosines remain unchanged. Direct sequencing of the PCR-products gives the average methylation status of a population of molecules; cloning of the PCR-products and subsequent sequencing of different clones provides methylation maps of individual DNA-molecules. One Y, one Z, one YZ, three XYZ DNA samples (the same as used for the DNA gel blot analysis, hemizygous for all loci), and one sample hemizygous for X and homozygous for Y (referred to as XYY) were treated with bisulfite (see "Materials and Methods"). Primers were designed to allow locus-specific amplification of the 5' half of the non-coding strands of the *gus* genes residing in loci Y or Z (i.e. 3' half of the *gus*-coding sequence), based on the different 3'-UTRs, namely 3'*chs* and 3'*nos*, respectively. In this way, approximately 850 bp located upstream of the translation stop codon could be analyzed for both loci (Fig. 5A).

The PCR products were cloned into the pGemT or pCR2.1 vectors and several randomly selected clones were sequenced, with each line representing the DNA methylation pattern from a different clone (Fig. 5A). The degree of methylation of cytosines in a different sequence context is given in Figure 5B. Locus Y appears to show a slight increase in cytosine methylation in CG context in the clones recovered from the YZ sample (Fig. 5B, 7% CG) compared to those from the Y sample (Fig. 5B, 1.4% CG), which is consistent with the weak methylation observed in the DNA gel blot analysis (Fig. 4B; lanes 5 and 6). The methylation of locus Y in XYZ samples is significantly increased in CG and CNG sites (Fig. 5B, 40% CG, 11% CNG), compared to the YZ sample (Fig. 5B, 7% CG, 1.4% CNG). Locus Y in the XYY sample is highly methylated in all sequence contexts (Fig. 5B, 97% CG, 83% CNG and 65% CNN). Also locus Z in the XYZ samples shows strong methylation in all sequence contexts (Fig. 5B, 92% CG, 88% CNG and 85% CNN) compared to the Z and YZ sample (Fig. 5B, 0% CG, 1.2% CNG and 0% CNN, and 1.2% CG, 1.8% CNG and 0.3% CNN, respectively). Together, these data suggest that both loci become differentially methylated upon inactivation by the silencing-inducing locus X.

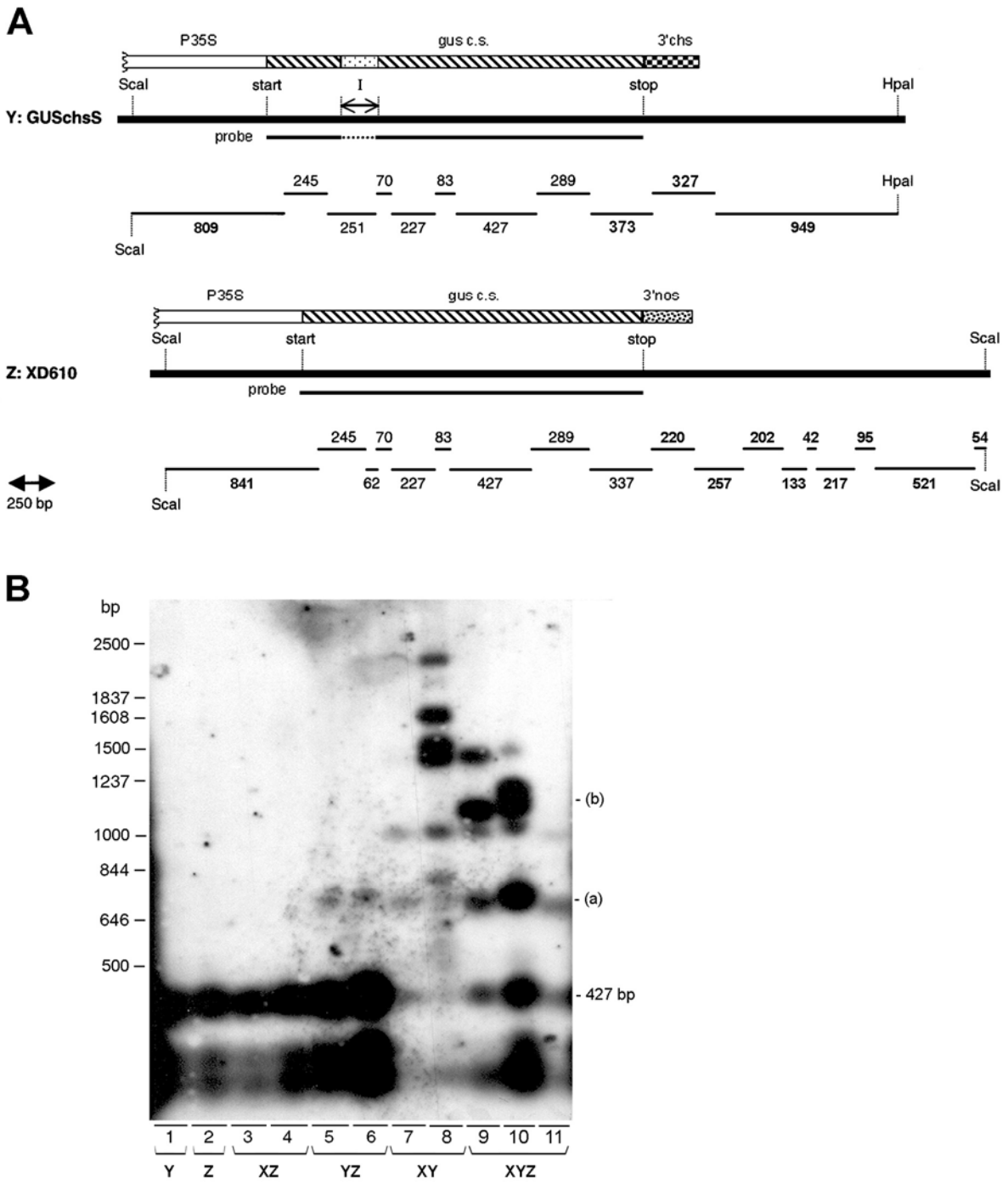

Figure 4. Outline of the DNA gel blot to analyze the cytosine methylation status in the *gus*-coding sequences of XYZ and YZ plants. **(A)** Representation of the analyzed restriction sites. The analyzed region of the *gus*-containing T-DNAs in locus Y and Z (GUSchs and LXD610, respectively) is represented by a thick black bar for each T-DNA. The relative location of the start and stop codons of the *gus* gene as well as the synthetic intron (I) are indicated above the bars. The recognition sites of the methylation-insensitive restriction enzymes *Hpa*I and *Sca*I were used to border the segments for methylation analysis. The functional elements of the *gus* chimeric genes are indicated by boxes above the black bars (for abbreviations, see Figure 1). The fragments drawn below the bars are the DNA fragments obtained upon full *Hpa*II digestion of the *Sca*I/*Hpa*I and *Sca*I/*Sca*I fragments of the T-DNAs in locus Y and Z,

respectively (size in bp). The sequence used as probe (the full-length *gus*-coding sequence) is given as a bar just below the Y and Z T-DNA fragments. **(B)** DNA gel-blot of tobacco plants containing different combinations of loci X, Y and Z. Genomic DNA of normal *gus*-expressing Y, Z, XZ and YZ hybrids and *gus*-silenced XY and XYZ hybrids (13 μg per lane) was cut in triple digests with the two methylation-insensitive enzymes *Hpa*I and *Sca*I and with the methylation-sensitive *Hpa*II. The full-length *gus*-coding sequence was used as probe. The length of size markers is indicated in bp. Specifically in XY and XYZ samples, hybridizing bands of higher molecular weight are detected. (a) and (b) indicate bands of approximately 0.7 kb, and 1.1 kb and 1.2 kb, respectively.

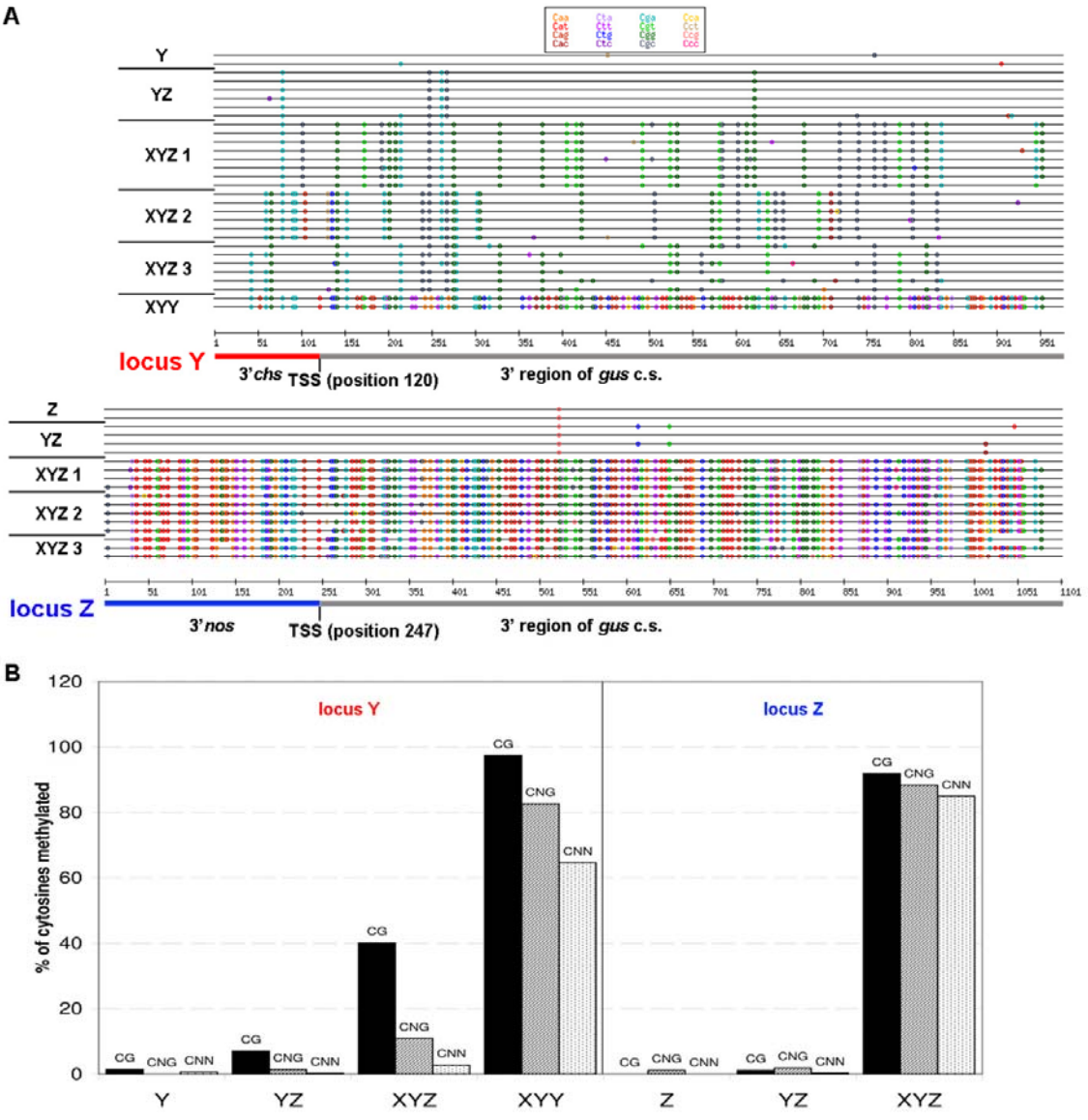

Figure 5. Distribution and density of cytosine methylation of loci Y (red) and Z (blue) in one Y, one Z, one YZ, three XYZ and one XYY tobacco plant. **(A)** Genomic bisulfite sequencing of the non-coding strands of locus Y (upper pane, red) and Z (upper pane, blue), showing the methylation distribution along approximately 850 bp upstream of the translation stop codon of the *gus*-coding sequences in different genetic backgrounds. The sequences obtained were processed through the MethTools software (Grunau *et al.*, 2000). The colored dots indicate the positions of the methylated cytosines in trinucleotide contexts.

The red and blue bars indicate the position of the 3'-UTRs of locus Y (3'*chs*) and Z (3'*nos*) repectively, the grey bars that of the 3' region of the *gus*-coding sequence. c.s., coding sequence; TSS, translation stop site. **(B)** Percentages of methylated cytosines in symmetrical (CG, CNG) and non-symmetrical (CNN) contexts for locus Y and Z in different genetic backgrounds. Data for locus Y were assembled from two Y, six YZ, twenty XYZ and two XYY clones, and for locus Z from two Z, four YZ and twelve XYZ clones.

Discussion

According to the current model for PTGS or RNA silencing (Kooter *et al.*, 1999; Matzke *et al.*, 2001; Vance and Vaucheret, 2001), dsRNA molecules elicit the activation of the silencing response (Kooter, 2005). Because locus X consists of two invertedly repeated T-DNAs that contain convergently transcribed *npt*II transgenes (Van Houdt *et al.*, 2000a), we postulate that this locus induces silencing either by sporadic read-through transcription over the T-DNA right border sequences or by disturbed transcription as a result of possible secondary structures formed near the centre of the IR. The produced aberrant transcripts are believed to be the substrate for RDR6-dependent dsRNA synthesis (Gazzani *et al*, 2004; Wassenegger and Pélissier, 1998). Consistently, PTGS induced by IR loci has been shown to depend on RDR6 in *Arabidopsis* (Butaye *et al,* 2004). Subsequent cleavage of the dsRNA molecules originating from locus X by a Dicer-like RNase would lead to the production of primary siRNAs. Indeed, we show that low-molecular weight RNA fractions of locus X-harboring tobacco plants, and not similar fractions of transgenic plants with normal *npt*II expression, contain approximately 22-nt small *npt*II RNAs (Fig. 3A). The post-transcriptionally silenced transgene locus X can silence *in trans* the partially homologous *gus* transgene in locus Y (Van Houdt *et al.*, 2000a; Table 1). The mechanism of *in trans*-silencing is most probably based on the presence of siRNAs that correspond to the region of homology between silencing inducer and target RNA, namely the 3'*chs* region in our analysis. Thus, the 3'*chs*-specific siRNAs seem to mark the locus Y-derived *gus* transcripts for degradation.

How can locus X direct degradation of locus Z-derived *gus* transcripts in XYZ plants? According to the current model of target RNA degradation (Elbashir *et al.*, 2001a), the likely hypothesis is that siRNAs homologous to locus Z-derived transcripts would be involved. Indeed, *gus*-specific siRNAs are readily detected in the low-molecular weight RNA fraction of XYZ plants, but not in similar fractions of non-silenced YZ plants (Fig. 3B). These molecules are candidates to function as siRNAs for sequence-specific degradation of *gus* transcripts, resulting in the low GUS activity detected in XYZ tobacco plants compared with that in Z plants. In a recent study, the spreading of RNA targeting upon virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* and *Arabidopsis* has been described (Vaistij *et al.*, 2002). The effect of

VIGS spreads beyond the viral sequences inducing RNA silencing, because the single-stranded target transcripts are converted to dsRNA by the putative SDE1/SGS2/RDR6 (Vaistij *et al.*, 2002). This observation gives an insight into how the detected *gus*-specific siRNAs, corresponding to the most 3' 800 bp of the *gus*-coding sequence, might be generated. RDR6-dependent synthesis of *gus* antisense RNA could be primed by locus X-derived 3'*chs*-specific siRNAs on a locus Y-derived transcript, in analogy with the extension of primer siRNAs into a dsRNA product in embryo extracts of Drosophila (Lipardi *et al.*, 2001). Alternatively, because no priming is required in an RDR-dependent polymerization reaction (Schiebel *et al.*, 1993), a particular feature of the Y-derived *gus* transcript, such as the mere association with a siRNA-protein complex or partial degradation by an RNA-induced silencing complex (Hammond *et al.*, 2000), could allow it to be recognized by a RDR6 as template for synthesis. Subsequently, RNase III-like cleavage of the nascent dsRNA would give rise to the detected small *gus* RNAs, which are probably involved in the 3’ to 5’ spreading of silencing, as revealed by the reduced GUS activity in XYZ hybrids.

Several studies in plants indicate that silencing can also spread to transgene regions downstream of the primary target (5’ to 3’ spreading; Braunstein *et al.*, 2002; Han and Grierson, 2002; Vaistij *et al.*, 2002). This observation cannot be ascribed to siRNA-primed RDR-directed synthesis of dsRNA on the basis of transgenic mRNA targets, nor can it be reconciled easily with unprimed RDR synthesis, which appears to start preferentially at the 3' terminus of the template (Schiebel *et al.*, 1993).

Although several papers describe the production of secondary siRNAs and the spreading of RNA silencing induced upon viral infection (Braunstein *et al.*, 2002; Vaistij *et al.*, 2002) or by nuclearly expressed transgenes (Han and Grierson, 2002), target-specific siRNA production apparently does not occur by default. In tobacco plants transformed with a chimeric transgene comprising sequences encoding *gus* followed by satellite RNA (satRNA), there is no indication for spreading of siRNA production upon helper virus infection (Wang *et al.*, 2001). Also phytoene desaturase and ribulose-1,5-bisphosphate carboxylase endogenous transcripts do not serve as templates for secondary siRNA production upon VIGS in *Arabidopsis*, and are therefore not involved in the spreading process of RNA silencing (Vaistij *et al.*, 2002). In summary, the generality, requirements and characteristics of secondary siRNA production in RNA silencing remain to be determined.

PTGS in plants, resulting in the degradation of homologous RNAs, has frequently been associated with sequence-specific *de novo* methylation of transcribed sequences of silenced transgenes. Also *in trans*-silenced transgenes homologous to a silencing inducer become extensively methylated (Van Houdt *et al.*, 2000a; Béclin *et al.*, 2002). The region of methylation of a silenced transgene, induced upon viral infection, has been confined to the region of

homology between the viral genome and the transgene (Jones *et al.*, 1998; Wang *et al.*, 2001). However, in other studies methylation of a silenced transgene induced upon viral infection spreads into transcribed sequences not corresponding to viral sequences (Jones *et al.*, 1999; Thomas *et al.*, 2001; Vaistij *et al.*, 2002), which has been associated with maintenance of silencing in the absence of the viral inducer (Vaistij *et al.*, 2002). We observed enhanced cytosine methylation of the silenced *gus* gene(s) in XY and XYZ plants via DNA gel blot analysis, whereas the non-silenced *gus* gene(s) in Y, Z, XZ and YZ plants remain(s) hypomethylated (Fig. 4B). Bisulfite genomic sequencing results suggest that the Y *gus* gene in XYZ plants is predominantly methylated in CG sites (Fig. 5). Similarly, the *in cis*- and *in trans*-methylation induced by locus 1 (= locus X) of the homologous locus 2 (Van Houdt *et al.*, 2000a), consisting of a single insert of the same GVCHS287 T-DNA as locus 1, occurs predominantly in symmetrical cytosines (Kovařík A., personal communications; Fojtova *et al.*, 2006; see further in this Chapter, Section 2). Methylation of CG sites in locus Y is maintained after segregation of the silencing-inducing locus 1 (S1 generation), and even becomes denser and spreads from the 3' to 5' region of the *npt*II gene. In the S2 generation, stochastic silencing is observed with variable degrees, but can reach an efficiency of 100% (Kovařík A., personal communications). Moreover, tissue culture of tobacco plants hemizygous for locus 1 induces spreading of methylation from the IR center to the 5’ half of the *npt*II gene and the promoter region, resulting in transcriptional gene silencing (TGS) of locus 1 (Fojtova *et al.*, 2006; see further in this Chapter, Section 2). We also observed a small increase in CG methylation in YZ plants that are segregants from an XYZ plant (Fig. 5B). After some generations, this methylation might also spread further to the 5’ end of the *gus* gene and possibly to the promoter region. Spreading of methylation to upstream sequences over generations could account for the hypermethylation observed in a descendant of a self-fertilized hemizygous XY plant (Fig. 4B) and in the XYY plant (Fig. 5), which is consistent with the hypermethylation of cytosines in all sequence contexts in the 5’ region of the *npt*II genes in the transcriptionally silenced epiallele of locus 1 (Fojtova *et al.*, 2006; see further in this Chapter, Section 2). Based on the GUS activity measurement in a protein extract from the XYY plant, we can however not distinguish whether the observed *gus* silencing results from TGS of locus Y or PTGS induced by locus X. It would be interesting to see whether this spreading along Y sequences also occurs in subsequent generations of heterogenous YZ and XYZ plants.

In contrast to the CG methylation observed for locus Y, the Z *gus* gene appears to show heavy methylation in both symmetrical (CG and CNG) and asymmetrical (CNN) context. The mechanism that would discriminate between both *gus* sequences during the process of DNA methylation remains however unclear. In *Arabidopsis thaliana*, the establishment of DNA methylation in all sequence contexts is entirely dependent on DRM1/DRM2 (Cao *et al.*, 2003;

Cao and Jacobsen, 2002), which can be targeted by siRNAs generated from different RNA silencing pathways (see Chapter 1, Section 1.3.; Chan *et al.*, 2005). Maintenance however depends on different methyltransferases and is locus-specific. The maintenance of CG methylation after removal of the silencing trigger requires MET1 (Aufsatz *et al.*, 2004; Jones *et al.*, 2001). Maintenance of non-CG methylation depends on CMT3 (Bartee *et al.*, 2001) and DRM1/DRM2 (Chan *et al.*, 2004), with varying degrees of redundancy, and possibly requires an active signal for propagation during each cell cycle. A good candidate for such a signal is the persistent generation of siRNAs corresponding to the methylated sequence. The differential methylation patterns for locus Y and Z could be due to the different ways both loci are silenced. Locus Y is silenced *in trans* by primary *chs*-specific siRNAs derived from locus X, whereas locus Z is transitively silenced by secondary siRNAs originating from locus Y. Methylation of locus Y induced by the *chs*-specific siRNAs might be followed by spreading of mainly CG methylation into the non-homologous *gus* sequence of locus Y, which has been reported by others (Jones *et al.*, 1999; Kovařík A., personal communications; Thomas *et al.*, 2001; Vaistij *et al.*, 2002). In contrast, the secondary *gus*-specific siRNAs could induce methylation of locus Z in all sequence contexts. Alternatively, methylation of locus Y and Z could be both established in all contexts by DRM1/DRM2, but maintenance could be different as a result of different features of secondary siRNAs produced by locus Y and tertiary siRNAs originating from locus Z (García-Pérez *et al.*, 2004), binding either MET1 (CG maintenance) or CMT3 (non-CG maintenance), respectively. In this scenario, the siRNAs would methylate *in cis* the locus from which they derive. A third explanation could be the difference in copy number of the *gus* genes in both loci Y and Z. Locus Y contains only one copy, whereas locus Z harbors two or more copies, possibly resulting in more methylating signals. Similarly, the XYY plant homozygous for locus Y shows enhanced cytosine methylation (Fig. 5). Besides RdDM, *de novo* methylation of the *gus* sequences might also be induced by DNA-DNA interactions. DNA pairing between the methylated 3'*chs* regions of the *npt*II genes of the silencing-inducing locus X and target Y could be a signal for methylation of the paired sequences, followed by spreading of methylation into the non-homologous *gus* sequences. Subsequent pairing of the Y and Z *gus* genes would result in methylation of the Z gene. However, the differential methylation patterns of both loci Y and Z and the observation that the weak methylation of the Y *gus* gene in YZ samples is not imposed on the Z *gus* gene contradict this hypothesis.

We cannot exclude the possibility that the differential methylation patterns observed for locus Y and Z result from experimental artifacts associated with bisulfite genomic sequencing. First, it could be that some highly methylated Y DNA molecules indeed are present in XYZ samples, but that a possible amplification bias towards molecules with a low CG% (Warnecke *et al.*, 1997) results in the observed methylation pattern of predominant symmetrical methylation

for Y (Fig. 5B). Second, locus Z could indeed be highly methylated in all sequence contexts in XYZ plants, although it cannot be excluded that the cytosines in the sequenced molecules do not represent *bona fide* methylated, but rather unconverted non-methylated cytosines. However, DNA isolation, bisulfite treatment, and subsequent PCR amplifications were carried out in triplicate, and these same samples do show nice conversion of non-methylated cytosines in locus Y.

The occurrence of transitive silencing will have to be taken into account in case RNA silencing or RNAi is the technique of choice in functional genomic studies to obtain a null mutant phenotype for any particular gene (Nishikura, 2001). It is possible that particular siRNAs produced by a silencing inducer correspond to an identical stretch of nucleotides in a family member of the studied gene, not targeted on purpose, what could initiate a process in which the partially homologous endogenous transcript is used as template to produce secondary siRNAs (Sanders *et al.*, 2002). Therefore, transitive silencing of coordinately expressed genes with highly conserved domains can be anticipated. However, several studies have failed to demonstrate spreading of silencing along endogenous sequences (Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005; Vaistij *et al.*, 2002). The design of a dsRNA trigger will be crucial to create a selected gene-specific mutant phenotype. On the other hand, we envision that transitive silencing could be applied in silencing technologies, circumventing laborius construction of inverted repeat transgenes.

Materials and Methods

Transgene tobacco lines and production of hybrid plants

The production of plants containing locus X and several characteristics of locus X have been described previously (locus 1 in Van Houdt *et al.*, 2000a, 2000b). The locus Y-containing primary tobacco transformant GUSchsS29 was obtained via *Agrobacterium tumefaciens* cotransformation of *Nicotiana tabacum* (L.) cv. Petit Havana SR1 leaf discs with the *Agrobacterium tumefaciens* strains C58C1RifR(pGV2260, pNE) and C58C1RifR(pGV2260, pGUSchsS). The plasmids pNE, carrying a hygromycin resistance marker (De Buck *et al.*, 1999) and pGUSchsS with the phosphinothricin resistance marker (Van Houdt *et al.*, 2000b) have been described previously. Transformant GUSchsS29 was obtained upon hygromycin selection and, in addition to the pNE T-DNA insert(s), contained an independently segregating locus, designated locus Y, harboring a single copy of the GUSchsS T-DNA. The tobacco leaf disc transformation in which the locus Z-containing primary transformant LXD610-2 was generated, has been described previously (De Loose *et al.*, 1995).

Hemizygous X and Z plants were obtained as hybrid progeny by crossing tobacco plants homozygous for locus X (=HOlo1) and homozygous for locus Z (=LXD610-2/9) to wild-type SR1, respectively. Hemizygous Y plants originated from the cross between the hemizygous primary tobacco transformant GUSchsS29 and SR1 and by selecting for the presence of locus Y in the hybrid progeny with phosphinothricin (10 μg/ml). Hemizygous plants XY and YZ were the hybrid progeny plants of the cross between HOlo1 and GUSchsS29 and between GUSchsS29 and

LXD610-2/9, respectively, which were selected for the presence of Y. Hemizygous plants XZ were the hybrid progeny of the cross between HOlo1 and LXD610-2/9 and hemizygous plants XYZ were obtained by crossing hemizygous plants XY to LXD610-2/9, which is homozygous for locus Z, and selected for the presence of locus Y. Because the presence of locus X in this hybrid progeny could not be selected for, we screened for hemizygous plants XYZ through a locus X-specific polymerase chain reaction.

Enzymatic assays

Protein extracts were prepared and GUS activity measured as described by Van Houdt *et al.* (2000b).

DNA gel-blot analysis

Genomic DNA from leaf tissue of mature tobacco plants was isolated with the DNeasy plant kit (Qiagen, Hilden, Germany). DNA gel blot hybridization was mainly done as described previously (Van Houdt *et al.*, 1997). Probes were labeled with the Gene Images random prime labeling kit (Amersham Biosciences, Little Chalfont, UK) and detected with the Gene Images CDP-Star module (Amersham Biosciences).

Bisulfite genomic sequencing of the 3' half of the gus-coding region

Bisulfite sequencing was performed according to the protocol described by Grunau *et al.* (2001). Total genomic DNA was digested with an excess of the methylation-insensitive restriction enzyme *Kpn*I. Of the digested DNA, 500 ng supplemented with 10 µg of carrier tRNA (Sigma-Aldrich) was denatured in 0.3 M NaOH at 42°C for 20 min. A fresh bisulfite solution was prepared by dissolving 5.41 g sodium bisulfite (Sigma-Aldrich) in 8 ml of distilled water without vigorous shaking. After adding 500 µl of 20 mM hydroquinone (Sigma-Aldrich), the solution was adjusted to pH 5 with 10 M NaOH, and the final volume was adjusted to 10 ml with distilled water. After complete dissolution of the bisulfite, the solution was passed through a 0.22-µm filter, and 1200 µl was added directly to the denatured DNA. The reaction was overlaid with 200 µl of mineral oil (Sigma-Aldrich) and incubated in the dark at 55°C for 4 h. The DNA was desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI) according to the manufacturer's protocol. The DNA was eluted in 110 µl of 10 mM Tris-HCl (pH 7.5), of which 100 µl was desulfonated with 11 µl of 3 M NaOH at 37°C for 20 min. The desulfonated DNA was neutralized with 47 µl of 10 M ammonium acetate, supplemented with 10 µg of tRNA and precipitated with 500 µl of 95% ethanol at -20°C overnight. The DNA was resolved in 100 µl 10 mM Tris-HCl (pH 7.5) and stored at -20°C. The bisulfite-treated DNA was amplified by semi-nested PCR in a 50 µl reaction volume containing 1x PCR Rxn Buffer (-MgCl2; Invitrogen, Carlsbad, CA), 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM forward primer, 1 µM reverse primer, 5 U Taq DNA polymerase (Invitrogen), and 2.5 µl of template (bisulfite-treated DNA or first PCR). For amplification of the 5' half of the non-coding strand of the Y *gus* gene, the first forward primer 5'-YGTTACAAGAGAGAAATCGYYAATATAG-3' and the second forward primer 5'-GGAAATAAGAAAATAATTGAATTTGGGG-3', specific for the 3'*chs*-UTR sequence, and for amplification of the 5' half of the non-coding strand of the Z *gus* gene, the first forward primer 5'-AGTTTGYGYGYTATATTTTGTTTT-3' and the second forward primer 5'-ATYGYGTATTAAATGTATAATTGYGGG-3', specific for the 3'*nos*-UTR sequence, were used together with the first reverse primer 5'-ACCAAAAACCACACACAATATAATATCTACCC-3' and the second reverse primer 5'-AACTCAATTAAAACCAACTCCTACC-3' were designed. The PCR program consisted of 2 min of initial denaturation at 94°C followed by 5 cycles of 1 min at 94°C, 2 min at 50°C, 3 min at 72°C, subsequently followed by 25 cycles of 30 s at 94°C, 2 min at 50°C, 90 s at 72°C. The program was ended with an extension step

for 10 min at 72°C. The PCR products were purified with the High Pure Purification Kit (Roche Diagnostics, Brussels, Belgium) according to the manufacturer's instructions. The amplicons were eluted in 60 µl elution buffer. After subcloning in pGEM-T (Promega) or pCR2.1 (Invitrogen), at least nine clones from each cloned amplicon were selected for DNA isolation and sequencing.

Small RNA analysis

To detect small RNAs, the procedures described by Hamilton and Baulcombe (1999) and Mette *et al.* (2000) were adapted. Tobacco leaf tissue was frozen in liquid nitrogen and total RNA was extracted with TRIzol reagent (Life Technologies, Paisley, UK), according to the manufacturer's instructions. Most of the high-molecular weight RNAs were precipitated and the lower molecular weight RNAs were recovered from the supernatant as described. For the different samples analyzed, a similar amount of RNA of the lower molecular weight RNA fraction, as estimated on gel, was separated on gel (15% (v/w) polyacrylamide, 7 M urea) and transferred to Hybond N+ membranes (Amersham Biosciences) by electroblotting with a Kem en Tec semi-dry blotter II (BIOzym, Landgraaf, The Netherlands). As size and polarity controls, DNA oligomers were loaded on the same gels. ^{32}P-labeled probes were synthesized *in vitro* from a linearized plasmid with an SP6/T7 transcription kit (Roche Diagnostics, Brussels, Belgium) and [α-^{32}P]CTP. The probe was hydrolyzed into fragments of approximately 50 nt. Hybridization and washes were performed as described (Hamilton and Baulcombe, 1999; Mette *et al.*, 2000) at 30°C. Labeled membranes were exposed to a Phosphorimager screen (Amersham Biosciences).

Distribution of materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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2. The epigenetic switch from post-transcriptional to transcriptional gene silencing is associated with changes in the distribution of methylcytosines along an invertedly repeated transgene locus

Abstract

Tobacco plants containing an inverted repeat (IR) transgene locus with two convergently transcribed neomycin phosphotransferase II (*npt*II) reporter genes show post-transcriptional gene silencing of the *npt*II genes. However, *in vitro* tissue culture induces epigenetic changes, resulting in a transcriptionally silenced epiallele of the IR locus. Here, we analyze the distribution and density of methylated cytosines in the promoter region and the 5' end of the *npt*II-coding sequence of both epialleles in more detail by bisulfite genomic sequencing. We demonstrate that the switch from post-transcriptional to transcriptional silencing was correlated with a dramatic increase in methylation of cytosines in symmetrical and non-symmetrical context located 500 bp upstream of the transcription start site and in the 5'-transcribed region.

Introduction

Gene or transgene activity can be silenced at the transcriptional (TGS) or post-transcriptional (PTGS) level. Promoters of transcriptionally silenced genes are inactive and, thus, transcription is blocked, whereas those of post-transcriptionally silenced genes are active, but the transcripts are degraded before the protein products can be made. The ability to communicate silencing information to homologous loci seems to be a characteristic feature of many transgene loci. When homologous promoters are involved, the silencing is referred to as *in trans*-TGS (Vaucheret and Fagard, 2001) and when the coding region is involved, as *in trans*-PTGS (Vaucheret *et al.*, 2001). Several observations suggest that TGS and PTGS may be mechanistically related (Mette *et al.*, 1999; Sijen *et al.*, 2001). In plants, both types of interactions are frequently associated with DNA methylation of the homologous regions.

Highly expressed and convergently transcribed transgenes, as can be found at inverted repeat (IR) loci, have particularly powerful silencing capacities *in cis* and *in trans* of homologous unlinked loci in both mammals and plants (Finnegan *et al.*, 1998; Garrick *et al.*, 1998; Matzke *et al.*, 2000; Stam *et al.*, 1998). The palindromic arrangement is important for the silencing capacities and methylation of the IR locus, which has been demonstrated in *Arabidopsis thaliana* by targeted deletion of one of the transgenes in the IR (De Buck and Depicker, 2001; De Buck *et al.*, 2001). The potent capacity of IR loci to methylate and silence homologous sequences *in trans* can be explained by two model mechanisms: (1) physical interaction of the IR and homologous sequences via DNA-DNA pairing, and (2) involvement of aberrant RNA molecules that result from disturbed transcription through the IR center. In petunia (*Petunia hybrida*), all the rare instances of PTGS caused by a promoterless chalcone synthase transgene were associated with both IR arrangement of the T-DNA and DNA methylation (Stam *et al.*, 1998). A complex enhancerless transgene locus could be transcribed only in the presence of the homologous locus that carried a functional enhancer (Matzke *et al.*, 2001), suggesting that unlinked transgenes might be paired in the plant nucleus. Pairing interactions have also been proposed to be involved in paramutation, i.e. heritable *in trans*-interacting effects of certain alleles in maize (Chandler and Stam, 2004). In parallel, elegant experiments with forced transcription of promoter or cDNA sequences arranged in an IR structure that produce double-stranded hairpin RNA (hpRNA), imply that also RNA can mediate *in trans*-silencing (Mette *et al.*, 1999). The *in trans*-TGS effect induced by hpRNA products was accompanied by *de novo* methylation of cytosines in symmetrical and non-symmetrical context, a pattern known to be a hallmark of RNA-directed DNA methylation described in viroids (Pélissier *et al.*, 1999). The powerful *in trans*-silencing capacity of IRs is not restricted to transgenes and has been observed for endogenous genes. For example, the strain Wassilewskija of *Arabidopsis* contains four

copies of a gene coding for the tryptophan biosynthesis, of which two are arranged as an IR and two as unlinked copies. All four genes are densely methylated and it was demonstrated that the IR locus triggers *de novo* methylation and silencing of the unlinked homologous loci (Luff *et al.*, 1999). Despite these observations, the presence of transgenes in IR arrangements was not sufficient in other transgenic systems to trigger the *in trans*-silencing process (Lechtenberg *et al.*, 2003; Meza *et al.*, 2002). For instance, *Arabidopsis* transgenes driven by the strong 35S promoter (P_{35S}) and arranged as IRs maintained high levels of expression. In this model system, PTGS was associated with dosage rather than with the arrangement of the transgenes (Lechtenberg *et al.*, 2003). However, it should be noted that in this case the inverted transgenes were located far from the center of the palindrome and that two other genes were located in-between the assayed P_{35S}-driven transgenes.

Palindromic sequence arrangements are frequently found in multicopy loci that also contain both tandem repeats and truncated T-DNA insertions and even vector sequences that make the contribution of each structure difficult to assess. Previously, we have described a tobacco (*Nicotiana tabacum*) transgenic locus 1 that contains two copies of a T-DNA organized as a near perfect IR with no or little non-homologous sequence in the center (Van Houdt *et al.*, 2000b). The residing P_{35S}:neomycin phosphotransferase II (*npt*II) reporter transgenes are organized in a tail-to-tail configuration within 1.8 kb from the IR center. Run-on analysis, experiments with inhibition of the chimeric potato (*Solanum tuberosum*) virus X:*npt*II replication, and dense methylation in the *npt*II-coding region indicated that the *npt*II genes at the IR locus 1 are post-transcriptionally silenced (Van Houdt *et al.*, 2000a; Van Houdt *et al.*, 2000b). During *in vitro* propagation in callus culture, the mode of transgene silencing was changed from post-transcriptional into transcriptional (Fojtová *et al.*, 2003). This event was accompanied by several changes in the distribution of the DNA methylation along the transgenes, hypermethylation of the 35S promoter being the most prominent. The novel epigenetic state was stably transmitted from the calli to regenerated plants and several plants carrying a TGS epimutant allele of locus 1 were recovered. Importantly, the PTGS or TGS epigenetic states were also stably transmitted to the next generation and, thus, are meiotically stable (Fojtová *et al.*, 2003).

Here, we have characterized the methylation patterns of the promoter region and the 5' end of the *npt*II-coding sequence of the TGS and PTGS epialleles of locus 1 in more detail. These regions showed heavy methylation of cytosines in symmetrical and non-symmetrical context in the transcriptionally silenced epiallele, whereas in the post-transcriptionally silenced epiallele the promoter was not significantly methylated and the cytosines in the 5' transcribed region were less methylated in CNG and non-symmetrical context, compared to those at CG sites.

Results

The transgenic locus 1 (locus X, Fig. 1, p.43) in the line hemizygous for locus 1 (HeLo1) contains two copies of the GVchs287 T-DNA arranged as an IR with the residing *npt*II genes silenced at the post-transcriptional level (Van Houdt *et al.*, 2000b). The isogenic line hemizygous for locus 1E (HeLo1E) contains an epimutated variant of locus 1, designated locus 1E, silenced at the transcriptional level. Previous results obtained by DNA gel blot hybridization indicated differential methylation of the locus 1 TGS and PTGS epialleles (Fojtová *et al.*, 2003). However, this technique allows only the detection of methylated cytosines located in restriction enzyme target sites. To obtain more detailed information about the distribution and density of methylated cytosine residues, we carried out a bisulfite genomic sequencing. Total genomic DNA from the HeLo1 and HeLo1E lines was treated with bisulfite. Primers were designed to allow amplification of the *npt*II-coding strand of the T-DNA, approximately 570 bp and 400 bp upstream and downstream of the transcription start site, respectively (Fig. 6A). The PCR products were cloned into the pGemT or pCR2.1 vectors and several randomly selected clones were sequenced (Fig. 6A), with each line representing the DNA methylation pattern from a different clone. The degree of methylation of the cytosines in a different sequence context is given in Figure 6B. In the clones recovered from the TGS epiallele, both the promoter and the transcribed region were methylated in all sequence contexts, i.e. symmetrical CG and CNG and non-symmetrical CNN. In contrast, in the clones from the PTGS epiallele, the sequences upstream of the transcription start site were not significantly methylated at any motif, while the immediate downstream region was methylated primarily at CG motifs. Although sequence identity does not allow left and right part of the IR to be analyzed separately by genomic sequencing, the relative homogeneity of the sequenced clones suggests that both parts of the repeat are methylated to a similar extent.

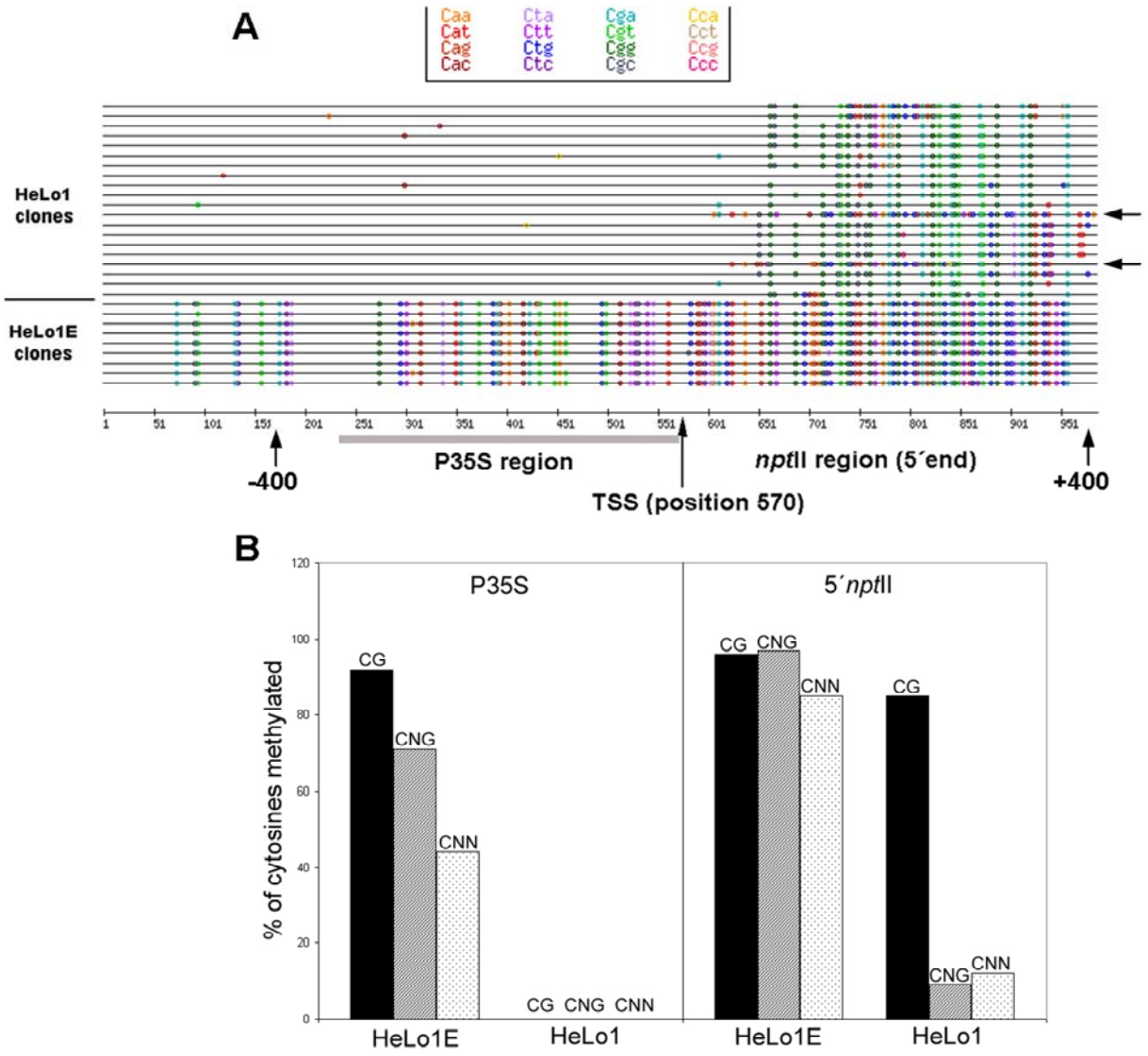

Figure 6. Distribution and density of cytosine methylation in the posttranscriptionally (HeLo1) and transcriptionally (HeLo1E) silenced epialleles of tobacco. **(A)** Genomic sequencing showing the methylation distribution along the 35S promoter and the 5'-*npt*II-coding region. The sequences obtained were processed through the MethTools software (Grunau *et al.*, 2000). The colored dots and horizontal grey bar indicate the positions of the methylated cytosines in trinucleotide contexts and the position of the core 35S promoter, respectively. TSS, transcription start site. The two horizontal arrows indicate the two HeLo1 clones with a high level of methylation, mentioned in the text. **(B)** Percentages of methylated cytosines in symmetrical (CG, CNG) and non-symmetrical (CNN) contexts for the promoter (-400/+1) and the 5'-*npt*II-transcribed region (+1/+400). Data were assembled from 21 HeLo1 and 9 HeLo1E clones.

Discussion

Methylation of the PTGS and TGS epialleles of locus 1 was studied by bisulfite genomic sequencing (Fig. 6). In the PTGS variant, the methylated region that started approximately 50 bp downstream from the transcription start site was sharply separated from the unmethylated upstream sequences and the promoter region. Only a few clones showed rare methylation in the promoter at a frequency corresponding to 1 mC/per clone/500 bp. Such a frequency might reflect a general methylation noise and probably does not influence the promoter activity. The cytosines in the 5' transcribed region were highly methylated at the CG sites, while those in CNG and non-symmetrical context were less methylated. Nevertheless, two HeLo1 clones (Fig. 6A, indicated with an arrow) had a high level of both symmetrical and non-symmetrical methylation, suggesting a certain epigenetic mosaicism among cells. Interestingly, in these densely methylated molecules, methylation spread apparently further upstream close to the transcription start site.

In contrast to the PTGS variant of locus 1, the TGS epiallele locus 1E was highly methylated in the promoter region: in the approximately 300 bp of the 35S core promoter, all clones displayed homogenous sequencing profiles and methylation appeared in all sequence contexts (92% CG, 71% CNG, and 44% CNN). This pattern is similar to that of the transcriptionally silenced 35S promoter in another tobacco (Park *et al.*, 1996) and petunia transgenic line (Meyer *et al.*, 1994), but clearly different from an inactive 35S promoter in *Arabidopsis* in which cytosines in non-symmetrical contexts were less methylated (Amedeo *et al.*, 2000). The two TGACG motifs at -83 and -71 that are important for binding of the activation sequence factor (Lam *et al.*, 1989) were completely methylated. The methylation density started to gradually decrease only at a distance of approximately 300 bp upstream from the transcription start site and the sites upstream of -500 were free of methylation. Also, the sequences downstream from the transcription start site were more heavily methylated in the TGS than in the PTGS epialleles. Together, these results suggest that the tissue culture-induced epimutation of locus 1 (Fojtová *et al.*, 2003) is caused by factors spreading the methylation and chromatin imprint from the IR center. Alternatively, *de novo* methylation could be specifically targeted to the promoter region after active recruitment of methyltransferases by transcription factors (Brenner *et al.*, 2005). The nature of the non-symmetrical methylation at the transcriptionally silenced promoter and the immediate downstream sequences remains enigmatic. This type of methylation is known to be mediated by RNA molecules. However, several lines of evidence argue against an RNA-directed mechanism of DNA methylation in the TGS epiallele. First, previous run-on assays (Fojtová *et al.*, 2003) and more sensitive quantitative PCR (M. Fojtová, unpublished results) failed to show transcription of the linked *npt*II

transgenes of the TGS locus 1E epiallele. Second, crossing experiments of the TGS HeLo1E to lines carrying homologous T-DNAs did not lead to *de novo* methylation of targets (Fojtová *et al.*, 2006), suggesting the absence of a diffusible signal emanating from a transcriptionally silenced 35S promoter. Perhaps the non-symmetrical methylation marks might rely on yet unidentified specific chromatin factors.

Materials and Methods

Plant material

The plants hemizygous for the PTGS locus 1 (HeLo1) were obtained by crossing a plant homozygous for locus 1 with an untransformed SR1 tobacco (Van Houdt *et al.*, 2000b). The line hemizygous for the TGS locus 1E was obtained by plant regeneration from long-term HeLo1 cell cultures as described in (Fojtová *et al.*, 2003).

Bisulfite sequencing of the P_{35S}-5'-nptII region

Bisulfite sequencing was performed according to the protocol described in the previous section. Total genomic DNA was isolated from lyophilized leaves or calli by a cetyltrimethylammonium bromide method as described previously (Kovařík *et al.*, 2000) and digested with an excess of the methylation-insensitive restriction enzyme *Mse*I to disrupt the IR structure of locus 1. For amplification of the P_{35S}-5'-*npt*II region, the same forward primer 5'-CATTACATCACCCATAATAAATACTTTCTC-3', the first reverse primer 5'-GAATAGAGAGAAAGATATATTTTTTAAGAT-3' and the second reverse primer 5'-GTAATAGAGATTGGAGTTTTTAAGAAAGTAG-3' were designed using mismatches at positions complementary to potentially methylated cytosines. In parallel, the same primer sequences were converted into degenerate primers: forward primer 5'-CATTRCATCARCCATRATRAATACTTTCTC-3', the first reverse primer 5'-GAAYAYAGAGAAAGATATATTTYTYAAGAT-3' and the second reverse primer 5'-GTAATAGAGATTGGAGYTYTTAAGAAAGTAG-3'. After subcloning in pGEM-T (Promega) or pCR2.1 (Invitrogen), at least nine clones from each cloned amplicon were selected for DNA isolation and sequencing.

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Chapter 4:

Down-regulation of endogene expression mediated by a transitive silencing signal in *Arabidopsis thaliana*

Submitted for publication:

Bleys, A., Van Houdt, H., and Depicker, A. Down-regulation of endogene expression mediated by a transitive silencing signal depends on the abundance of transgenic primary target transcripts.

Down-regulation of endogene expression mediated by a transitive silencing signal in *Arabidopsis thaliana*

Abstract

Some RNA silencing systems in plants, nematodes and fungi show spreading of silencing along target sequences, which is termed transitive silencing. Here, the question is addressed of whether endogenous targets can be silenced by a transitive silencing signal in plants. Transgenic *Arabidopsis thaliana* plants that harbour a silencing-inducing locus and a transgenic chimeric primary target showed silencing of a secondary transgenic target, and also exhibited down-regulation of the expression of the endogenous catalase genes coinciding with a knock-down phenotype. Strikingly, the efficiency of the catalase silencing appeared to be correlated with the zygosity of the primary target locus and, to a lesser extent, with that of the silencing-inducing locus. These data suggest that silencing of an endogene induced by transgenic secondary small interfering RNAs depends on the amount of primary target transcripts that can act as template for the production of an efficient transitive silencing signal.

Introduction

RNA silencing is a general term used for epigenetic regulatory pathways in which smRNAs of 21-26 nt mediate either transcriptional gene silencing by changes in chromatin state, or post-transcriptional gene silencing (PTGS) through target degradation or translational repression (for review, see Baulcombe, 2004). The mechanisms of these different silencing processes are very similar and evolutionarily conserved in most eukaryotic organisms, such as plants, animals and fungi (for review, see Meister and Tuschl, 2004). In the initiator step, (partially) dsRNA is produced that is subsequently processed into smRNAs by an RNase III-like enzyme (Dicer; Bernstein *et al.*, 2001; Zamore *et al.*, 2000). The smRNAs are incorporated into RNA-induced silencing complex (RISC)-like effector complexes that consist of at least an AGO protein, and guide chromatin modification, target degradation, or translational suppression in the final step of the process (Baumberger and Baulcombe, 2005; Béclin *et al.*, 2002; Fagard and Vaucheret, 2000; Hammond *et al.*, 2000; Morel *et al.*, 2002; Zilberman *et al.*, 2003). RNA silencing plays a biological role in gene regulation, genome integrity and defence against invading sequences, such as viruses, transposons and retroelements.

Since the accidental discovery that the introduction of a sense overexpressing transgene can lead to co-suppression of both the transgene and the endogene (Napoli *et al.*, 1990), RNA silencing has been widely used as a tool to down-regulate gene expression. Transgenic RNAs are recruited in the silencing pathway by the production of dsRNA. The RNA-dependent RNA polymerase RDR6 has been shown to be required for sense transgene triggered PTGS (Butaye *et al.*, 2004; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). How these sense RNAs are recognized by RDR6 is still unclear. Two models have been proposed. In the "threshold" model (Dougherty and Parks, 1995; Lindbo and Dougherty, 1992), a certain level of RNA might activate PTGS, whereas in the "aberrant RNA" model (Wassenegger and Pélissier, 1998; Waterhouse *et al.*, 1999), certain sense transgene loci would give rise to RNAs with some aberrant features. Both models could explain the PTGS observed in loci containing multiple copies of the transgene in direct (Ma and Mitra, 2002; Sijen *et al.*, 1996; Wang and Waterhouse, 2000) or inverted repeats (De Buck *et al.*, 2001; Stam *et al.*, 2000; Van Houdt *et al.*, 2000a, 2000b). Since dsRNAs have been shown to be potent inducers of PTGS (Fire *et al.*, 1998; Waterhouse *et al.*, 1998), hairpin constructs with self-complementary regions are frequently used to induce RDR6-independent co-suppression (Hilson *et al.*, 2004; Miki *et al.*, 2005; Smith *et al.*, 2000; Wesley *et al.*, 2001). The transgenic dsRNA is processed into siRNAs that mediate the degradation of target RNAs and possibly also DNA methylation (Hamilton and Baulcombe, 1999; Hamilton *et al.*, 2002; Mette *et al.*, 1999; Zilberman *et al.*, 2003). In plants, nematodes and fungi, not only trigger-derived siRNAs can be detected, but also siRNAs that correspond to regions upstream from the

region targeted by the dsRNA trigger. Moreover, in plants and fungi, siRNAs can be found corresponding to the downstream region as well (Braunstein *et al.*, 2002; Han and Grierson, 2002; Klahre *et al.*, 2002; Nicolás *et al.*, 2003; Sanders *et al.*, 2002; Sijen *et al.*, 2001; Vaistij *et al.*, 2002; Van Houdt *et al.*, 2003). These secondary siRNAs have been shown to induce silencing of targets without homology to the silencing inducer (for review, see Bleys *et al.*, 2006). This phenomenon, designated transitive silencing, involves an amplification process catalyzed by RDR6 that synthesizes dsRNA by using target RNAs as template, either by priming of primary siRNAs (Lipardi *et al.*, 2001; Sijen *et al.*, 2001) or by a primer-independent mechanism that starts at the 3' end of the target RNAs (Petersen and Albrechtsen, 2005; Schiebel *et al.*, 1993; Tang *et al.*, 2003; Vaistij *et al.*, 2002).

In *Caenorhabditis elegans* and *Mucor circinelloides*, transitivity occurs along transcripts derived from transgenes and endogenes (Alder *et al.*, 2003; Nicolás *et al.*, 2003; Sijen *et al.*, 2001), which in plants seem to behave differently in relation to transitivity. Many studies have demonstrated spreading of silencing along transgene sequences, but failed to do so for endogenous transcripts (Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005; Vaistij *et al.*, 2002). The ability of endogenes to serve as template for the RDR6-mediated production of secondary siRNAs has been described only once (Sanders *et al.*, 2002). Because all these transitivity studies only investigated the ability of secondary siRNAs to induce silencing of transgenic secondary targets, it is not known whether they can target plant endogenes as well.

Here, we address the question of whether a transitive silencing signal can mediate silencing of endogenous targets. Previously, we have demonstrated in tobacco *(Nicotiana tabacum*) that a post-transcriptionally silenced inverted repeat (IR) transgene locus (X) is able to silence a non-homologous transgene (Z), when a stepwise homology is created by introducing a chimeric primary target (Y) with one region homologous to the silencing inducer X and a second upstream region homologous to the secondary target Z (Van Houdt *et al.*, 2003; Chapter 3). A similar XYZ system has been developed in *Arabidopsis thaliana* (L.) Heynh., allowing us to demonstrate that a transitive silencing signal produced by an amplification mechanism is able to trigger silencing of endogenous targets, and that the efficiency of silencing depends on the primary target abundance.

Results

Development of a transitive silencing system in Arabidopsis *as reported by suppression of a highly expressed* gus *gene*

To check whether a particular isolated IR transgene locus X_{21} (P35S-*npt*II-3'*chs*) in *Arabidopsis* was able to transmit its *in trans*-silencing capacity to a primary target locus Y, we analyzed the occurrence of transitive silencing of a secondary target locus Z_c containing a reporter gene (P35S-*gus*-3'*nos*; Fig. 1). For details of constructs, plant material and crosses, we refer to "Materials and Methods". Because in tobacco some truncated constructs used as primary target had been previously observed not to trigger transitive silencing of a secondary target (data not shown), two different Y vectors were used: Y_7 (P35S-*gus*-*npt*II-3'*chs*) with a full-length *gus* transgene and Y_9 (P35S-*gus*800-*npt*II-3'*chs*) with only the last 800 nt of the *gus*-coding sequence. For the sake of simplicity, genotype and zygosity of the transgenic loci in the different *Arabidopsis* plants were schematically annotated as follows: slashes separate the loci X, Y and Z; XX, YY or ZZ represent homozygosity for the respective loci; hyphens indicate hemizygosity, double hyphens azygosity, and an asterisk unknown zygosity with the locus either homo- or hemizygous.

X_{21}-/Y_7-/Z_cZ_c and X_{21}-/Y_9-/Z_cZ_c hybrid plants, obtained after crossing experiments (see "Materials and Methods"), were analyzed for the remaining GUS activity by a fluorometric assay. A 70- to 140-fold reduction in GUS activity was observed in 19 X_{21}-/Y_9-/Z_cZ_c (Fig. 2, B) and 11 X_{21}-/Y_7-/Z_cZ_c (Fig. 2, A) hybrid plants, respectively, while in the progeny of the parental plants (10 $X_{21}X_{21}$/--/Z_cZ_c, 17 --/Y_7*/Z_cZ_c and 16 --/Y_9Y_9/Z_cZ_c plants; Fig. 2, C, D and E, respectively), all but two plants had a normal high GUS activity. These data indicate that not only in tobacco (Van Houdt *et al.*, 2003; Chapter 3), but also in *Arabidopsis*, *in trans*-silenced Y transcripts can produce a transitive silencing signal leading to spreading of silencing. This capacity does not depend on the transcript's potential to be translated into a functional protein, because frequency and degree of transitive silencing are very similar in the X_{21}-/Y_7-/Z_cZ_c (with Y_7 encoding a functional *gus* transgene) and X_{21}-/Y_9-/Z_cZ_c (with Y_9 encoding a truncated segment of the *gus* gene) hybrids. The reason for the lack of detected expression in two of the --/Y_9Y_9/Z_cZ_c plants was not investigated further, but it might be the induction of silencing *in cis* and *in trans* at low frequency when locus Y_9 and Z_c are present in homozygous condition.

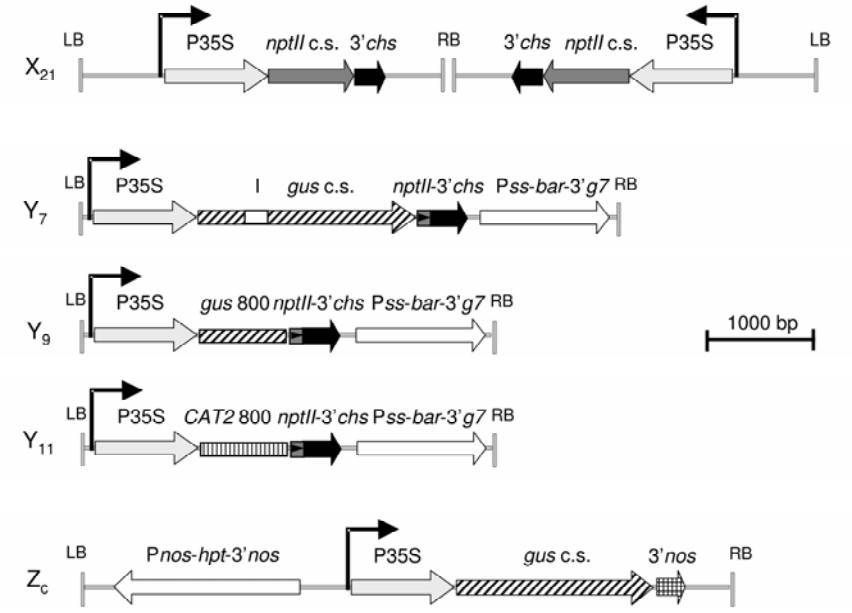

Figure 1. Schematic outline of the T-DNA constructs (drawn to scale), present in locus X_{21}, primary targets Y_7, Y_9 and Y_{11}, and secondary target Z_c. Silenced locus X_{21} contains two copies of the DEchs278 T-DNA that are arranged as an IR around the right border. Locus Z_c is a single copy locus. The copy number of the three Y loci is unknown. 3'*chs*, 3'-UTR of the chalcone synthase gene of *Antirrhinum majus*; 3'g7, 3'-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7; 3'*nos*, 3'-UTR of the nopaline synthase gene; *bar*, bialaphos acetyltransferase coding sequence conferring phosphinothricin resistance; *CAT2* 800, last 800 nt of the *A. thaliana CAT2* sequence; *gus* 800, last 800 nt of the *gus* sequence; *gus* c.s., *gus*-coding sequence; *hpt*, hygromycin phosphotransferase coding sequence; I, artificial intron; *npt*II c.s., neomycin phosphotransferase II coding sequence; P35S, CaMV 35S promoter; P*nos*, nopaline synthase promoter; P*ss*, promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase; LB, left T-DNA border; RB, right T-DNA border.

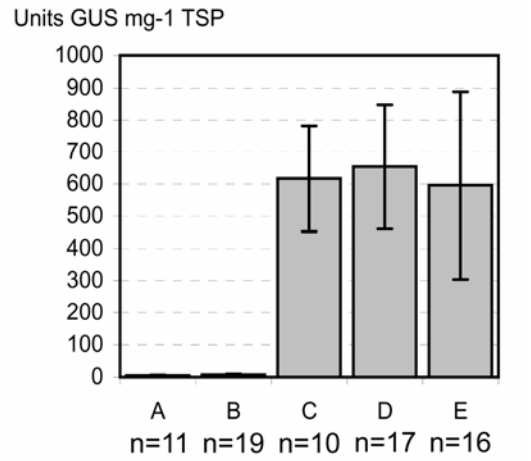

Figure 2. GUS activity levels in protein extracts of leaf tissue harvested from transgenic *Arabidopsis* plants containing different combinations of loci X_{21}, Y_7 or Y_9, and Z_c. SD is given by error bars. A, X_{21}-/Y_7-/Z_cZ_c; B, X_{21}-/Y_9-/Z_cZ_c; C, $X_{21}X_{21}$/--/Z_cZ_c; D, --/Y_7*/Z_cZ_c; E, --/Y_9Y_9/Z_cZ_c; n, total number of plants tested; TSP, total soluble protein.

Down-regulation of endogene expression mediated by a transitive silencing signal

Next, we wanted to determine whether the transitive silencing signal produced by the Y-derived transcripts was also able to trigger silencing of an endogenous target. We chose one of the *Arabidopsis* catalase genes as the secondary target. In *A. thaliana*, the catalase multigene family consists of three members (*CAT1*, *CAT2* and *CAT3*), with a nucleotide and amino acid sequence identity of 70-72% and 75-84%, respectively (Frugoli *et al.*, 1996). To target the *CAT2* gene, Y_{11} (P35S-*CAT*800-*npt*II-3'*chs*) was constructed, which is similar to P35S-*gus*800-*npt*II-3'*chs* in the Y_9 locus, but harbours the last 800 nt of the *CAT2*-coding sequence instead of the *gus* sequence. Because the transgenic locus Z_c, which is genetically linked to locus X_{21}, was not targeted in this experiment, it was omitted from the schematic representation in the discussion below. X_{21}-/Y_{11}-/*CATCAT* F1 hybrid plants (see "Materials and Methods") were analyzed together with progeny plants from the parental $X_{21}X_{21}$/--/*CATCAT* and --/$Y_{11}Y_{11}$/*CATCAT* plants, wild-type Columbia 0 (Col-0) plants and plants from two hairpin RNA (hpRNA)-silenced lines (CAT2HP1 and CAT2HP2; Vandenabeele *et al.*, 2004).

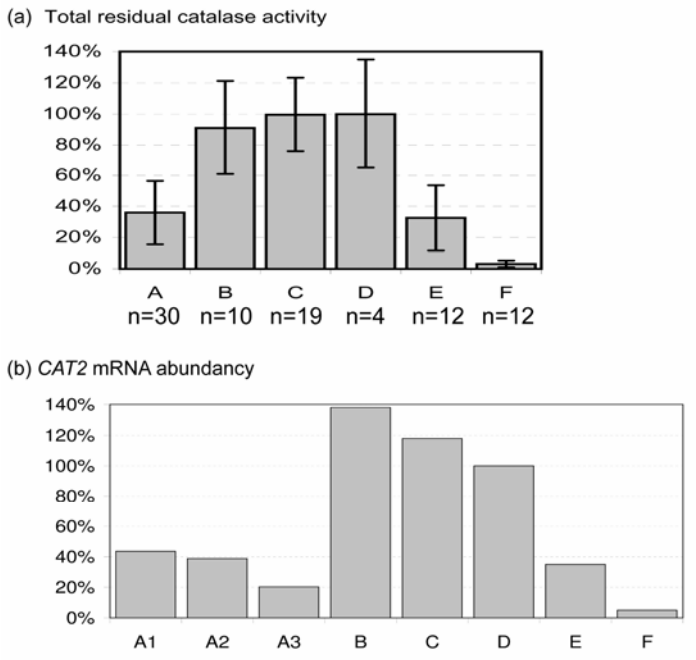

Figure 3. Protein and RNA levels in F1 hybrid plants, parental plants and two hairpin-silenced lines. **(a)** Average total residual catalase activity in wild-type and transgenic *Arabidopsis* plants containing different combinations of loci X_{21} and Y_{11}, or containing a *CAT2* hairpin construct (Vandenabeele *et al.*, 2004). Catalase activity in protein extracts of leaf tissue was measured with a spectrophotometric assay. The average total residual catalase activity of the different transgenic plants is relative to the average catalase activity of four wild-type (Col-0, D) plants, which is set at 100%. SD is given by error bars. A, X_{21}-/Y_{11}-/*CATCAT*; B, $X_{21}X_{21}$/--/*CATCAT*; C, --/$Y_{11}Y_{11}$/*CATCAT*; D, Col-0; E, CAT2HP1; F, CAT2HP2; n, total

number of plants tested. **(b)** *CAT2* mRNA abundancy. RNA was extracted from leaf tissue, first-strand cDNA was generated with an oligo(dT)$_{12-18}$ primer, and a quantitative real-time PCR was performed using *CAT2*-specific primers to determine the *CAT2* mRNA level relative to that in a wild-type background (100%). A1, A2 and A3, RNA levels in three X_{21}-/Y_{11}-/*CATCAT* plants; B, RNA level in one $X_{21}X_{21}$/--/*CATCAT* plant; C, RNA level in one --/$Y_{11}Y_{11}$/*CATCAT* plant; D, RNA levels in one wild-type Col-0; E, RNA level in one CAT2HP1 plant; F, RNA level in one CAT2HP2 plant.

All wild-type (Fig. 3a, D) and parental plants ($X_{21}X_{21}$/--/*CATCAT* and --/$Y_{11}Y_{11}$/*CATCAT*; Fig. 3a, B and C, respectively) retained a normal catalase activity, whereas the X_{21}-/Y_{11}-/*CATCAT* (Fig. 3a, A) plants had an average total residual catalase activity of 36%. This level of silencing corresponds with that observed in the CAT2HP1 line (Fig. 3a, E; 33% residual catalase activity), but is less strong than the reduction seen in the CAT2HP2 line (Fig. 3a, F; 3% residual catalase activity). To analyze the silencing at the RNA level, we performed a quantitative real-time PCR with a *CAT2*-specific primer pair (see "Materials and Methods"). Total RNA was prepared from leaf material and first-strand cDNA was generated with an oligo(dT)$_{12-18}$ primer. Figure 3b clearly shows a 2- to 5-fold reduced *CAT2* transcript level in X_{21}-/Y_{11}-/*CATCAT* (Fig. 3b, A1, A2 and A3) plants, compared to that of wild-type (Fig. 3b, D). A similar reduction could be observed in a CAT2HP1 plant (Fig. 3b, E), whereas the CAT2HP2 plant was 20-fold reduced (Fig. 3b, F). The parental plants ($X_{21}X_{21}$/--/*CATCAT* and --/$Y_{11}Y_{11}$/*CATCAT*; Fig. 3b, B and C, respectively) showed a slight increase in the *CAT2* RNA level. These results demonstrate that an *in trans*-silenced transgenic primary target can produce transitive silencing signals that down-regulate the expression of an endogene.

Dosage dependency for the primary target

To investigate the stability of endogene suppression via a transitive signal and to determine whether the efficiency of silencing is affected by the homozygous condition of either the silencing-inducing locus X_{21} or the primary target locus Y_{11}, seeds from a self-fertilized F1 hybrid plant (X_{21}-/Y_{11}-/*CATCAT)* were grown on selective medium for both X_{21} and Y_{11}. The two loci segregated independently and these F2 progeny plants were expected to have either genotype $X_{21}X_{21}$/$Y_{11}Y_{11}$/*CATCAT*, X_{21}-/$Y_{11}Y_{11}$/*CATCAT*, $X_{21}X_{21}$/Y_{11}-/*CATCAT* or X_{21}-/Y_{11}-/*CATCAT* at a 1:2:2:4 ratio. Thirty-six selected F2 progeny plants were analyzed and ranked according to their total residual catalase activity (Fig. 4a).

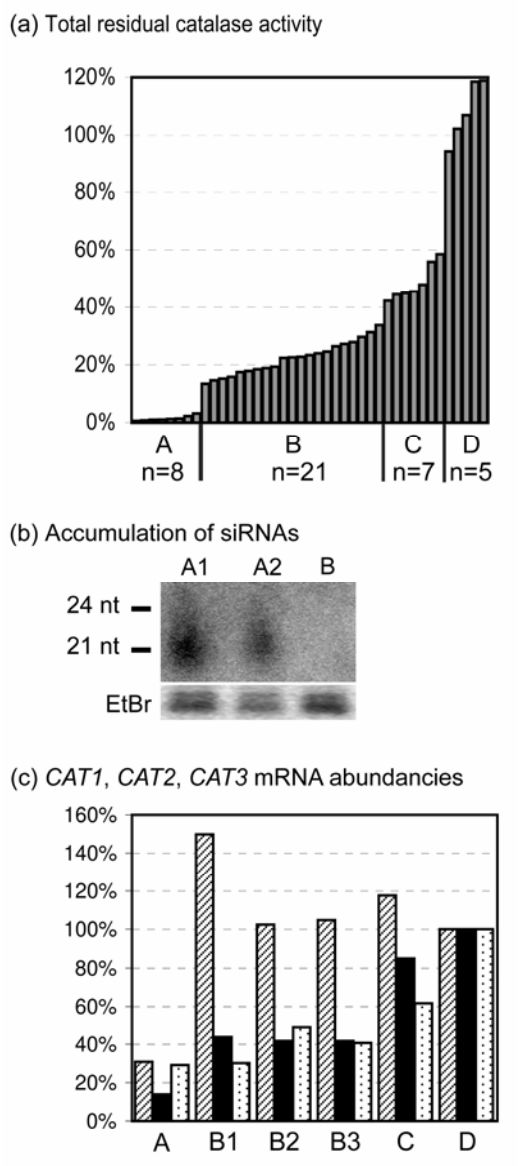

Figure 4. Protein levels, siRNA accumulation and RNA levels in F2 progeny plants and parental plants. **(a)** Total residual catalase activity in F2 progeny plants with segregating X_{21} and Y_{11} loci and parental --/$Y_{11}Y_{11}$/*CATCAT* plants. Catalase activity in protein extracts of leaf tissue was measured with a spectrophotometric assay. The total residual catalase activity of the different transgenic plants is relative to the average catalase activity of five wild-type plants (100%; not shown). A, X_{21}-/$Y_{11}Y_{11}$/*CATCAT* and $X_{21}X_{21}$/$Y_{11}Y_{11}$/*CATCAT*; B, X_{21}-/Y_{11}-/*CATCAT*; C, $X_{21}X_{21}$/Y_{11}-/*CATCAT*; D, --/$Y_{11}Y_{11}$/*CATCAT*; n, total number of plants per group. **(b)** Accumulation of *CAT2*-specific siRNAs in two silenced $X_{21}X_{21}$/$Y_{11}Y_{11}$/*CATCAT* plants (A1 and A2) and absence of siRNAs in one non-silenced --/$Y_{11}Y_{11}$/*CATCAT* plant (B). Low molecular weight RNA was extracted from leaves of 4-week-old plants. RNA oligomers of 21 and 24 nt were used as molecular markers. siRNAs were detected with a hydrolyzed ^{32}P-labeled probe comprising 274 bp in the 3' end of the *CAT2* sequence. With this probe it is impossible to discriminate between siRNAs originating from the transgene Y_{11} or from the endogenous sequences. The predominant ethidium bromide (EtBr)-stained species in the low molecular weight RNA fraction are shown as loading controls. **(c)** *CAT1*, *CAT2* and *CAT3* mRNA abundancies. RNA was extracted from leaf tissue, first-strand cDNA was generated with an oligo(dT)$_{12\text{-}18}$ primer, and a quantitative real-time PCR was

performed with gene-specific primers to determine the RNA levels of *CAT1* (hatched bars), *CAT2* (black bars) and *CAT3* (dotted bars) relative to those in a wild-type background (100%). A, RNA levels in one $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* plant; B1, B2 and B3, RNA levels in three X_{21}-/Y_{11}-/*CATCAT* plants; C, RNA levels in one $X_{21}X_{21}/Y_{11}$-/*CATCAT* plant; D, RNA levels in wild-type Col-0.

To assess whether the observed large variation in silencing efficiencies was correlated with the zygosity of loci X_{21} and Y_{11}, the segregation of 36 plants was analyzed. The eight plants exhibiting very efficient silencing (less than 3% total residual catalase activity; Fig. 4a, A) were all plants homozygous for locus Y_{11} ($X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* and X_{21}-/$Y_{11}Y_{11}$/*CATCAT*), whereas the seven plants with the lowest degree of reduction (between 42 and 59% total residual catalase activity; Fig. 4a, C) were hemizygous for locus Y_{11}, but homozygous for locus X_{21} ($X_{21}X_{21}/Y_{11}$-/*CATCAT*) and the 21 plants with intermediate silencing efficiencies (between 13 and 34% total residual catalase activity; Fig. 4a, B) were hemizygous for both loci (X_{21}-/Y_{11}-/*CATCAT*). The relative frequencies of the F2 plants with a total residual catalase activity that was low (8 plants), high (7 plants) and intermediate (21 plants) were consistent with an expected ratio of 3:2:4 for the segregation mentioned above, where the ratios of plants homozygous for locus Y_{11} were taken together (χ^2 = 3.02). These results suggest that the zygosity of the primary target Y_{11} locus and, to a lesser extent, that of the silencing inducer X_{21} locus influence the efficiency of transitive silencing of the catalase genes. Because the catalase activity in --/$Y_{11}Y_{11}$/*CATCAT* plants (Fig. 4a, D) was as high as that in wild-type plants, this dosage dependency did not result from silencing effects caused by the homozygous condition of locus Y_{11}.

We also looked at the accumulation of siRNAs that are the key molecules of PTGS. An RNA gel blot with a hydrolyzed ^{32}P-labelled probe comprising 274 bp in the 3' end of the *CAT2* sequence, revealed the presence of *CAT2*-specific siRNAs in $X_{21}X_{21}/Y_{11}Y_{21}$/*CATCAT* (Fig. 4b, A1 and A2) plants, whereas in --/$Y_{11}Y_{11}$/*CATCAT* plants (Fig. 4b, B) no such molecules were detected. In accordance to other reports (García-Pérez *et al.*, 2004; Himber *et al.*, 2003; Kościańska *et al.*, 2005), these secondary siRNAs are mainly of the 21-nt class.

To assess whether the most efficiently silenced $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* progeny plants displayed the characteristic catalase-deficient necrosis phenotype, we performed a high-light (HL) treatment (see "Materials and Methods"). HL irradiation induces photorespiration that leads to accumulation of H_2O_2 in catalase-deficient plants, followed by active cell death (Chamnongpol *et al.*, 1998; Dat *et al.*, 2003; Vandenabeele *et al.*, 2004). All $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* progeny plants had visible signs of cell death within 8 h of HL irradiation, whereas wild-type control plants did not, even after 48 h of HL (Figure 5). In conclusion, transitive silencing elicits the same knock-down phenotype as hairpin-induced silencing.

Figure 5. Phenotypes of wild-type and $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* plants after exposition to high-light (HL) irradiation (1000 µmol m^{-2} sec^{-1}). Cell death was visible in catalase-deficient $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* plants within 8 h of HL irradiation, while in wild-type control plants this phenotype did not occur, even not after 48 h of HL.

Transitive silencing of different members of the catalase gene family

The *Arabidopsis CAT2* gene targeted by the Y_{11} construct is part of a gene family. All three members are highly expressed in inflorescences, but in leaves only the *CAT2* and *CAT3* transcripts accumulate to high levels (Frugoli *et al.*, 1996). The latter genes are regulated by the circadian clock: a peak in mRNA abundance occurs for *CAT2* and *CAT3* in early morning, and in late afternoon and evening, respectively (Zhong and McClung, 1996; Zhong *et al.*, 1994). With the catalase activity assay used above, it is not possible to discriminate between the three catalase proteins, because the total residual catalase activity is measured, implying that the higher total residual catalase activity measured in leaf extracts from X_{21}-/Y_{11}-/*CATCAT* and $X_{21}X_{21}/Y_{11}$-/*CATCAT* plants might not result from inefficient silencing of *CAT2*, but of *CAT1* and *CAT3*. To assay to which extent the three members were silenced in F2 progeny plants, we performed a quantitative real-time PCR with three gene-specific primer pairs. Total RNA was prepared from leaf material harvested in the morning. In wild-type samples, the *CAT1* and *CAT3* mRNAs were approximately 500 to 600 times, and 20 to 25 times less abundant than those of *CAT2*, respectively (data not shown). The low *CAT1* expression in leaves implies only a small contribution of the CAT1 protein in the total residual catalase activity measured from leaf extracts.

The transcript levels of *CAT1*, *CAT2* and *CAT3* in leaf tissue of F2 progeny plants relative to those of a wild-type plant are given in Figure 4c. In this graph, the mRNA abundancies of *CAT1*, *CAT2* and *CAT3* in the wild-type plant (Fig. 4c, D) are all three set at 100%. We

observed a 7-fold reduction in the *CAT2* RNA level in a $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* sample (Fig. 4c, A), a 2.3-fold reduction in three X_{21}-/Y_{11}-/*CATCAT* samples (Fig. 4c, B1, B2, B3) and no significant reduction in a $X_{21}X_{21}/Y_{11}$-/*CATCAT* sample (Fig. 4c, C), indicating that the reduction in *CAT2* RNA levels was correlated with the zygosity of locus X_{21} and Y_{11}. This observation is consistent with the dosage dependency observed at protein level. The transcript levels of *CAT3* also seemed to be reduced to the same extent as those of *CAT2*, whereas *CAT1* RNA levels were not significantly reduced, except in a plant homozygous for both loci X_{21} and Y_{11} (Fig. 4c, A). We can conclude that the higher total residual catalase activity observed in plants hemizygous for Y_{11} compared to plants homozygous for Y_{11} results from less efficient silencing of both *CAT2* and *CAT3* transcripts targeted by the secondary *CAT2*-specific siRNAs originating from Y_{11}. To obtain further confirmation, four $X_{21}X_{21}/Y_{11}Y_{11}$/*CATCAT* F_3 progeny plants were analyzed (data not shown). In all four plants, *CAT2* and *CAT3* transcript levels were strongly reduced, and *CAT1* RNA levels ranged from 23% to 62% relative to those in a wild-type background.

Discussion

In plants, secondary siRNAs have been shown to target transgenic and viral sequences (Sanders *et al.*, 2002; Vaistij *et al.*, 2002; Van Houdt *et al.*, 2003), but it has never been investigated whether endogenous sequences can be silenced by a transitive silencing signal. Using the XYZ system in *A. thaliana*, we demonstrate that the *in trans*-silenced transgenic target Y_{11}, which contains the last 800 nt of the *CAT2*-coding sequence, produces secondary siRNAs that are able to trigger silencing of the endogenous catalase gene family. In *C. elegans*, transitive RNA interference (RNAi) has also been shown not to be limited to transgenic targets. Injection of *gfp*-dsRNA into animals expressing the *unc-22*::*gfp* transgene produced the twitching phenotype that is characteristic of loss of *unc-22* expression (Sijen *et al.*, 2001). Strikingly, we observe that the degree of transitive silencing depends on the DNA level of the primary target. In plants homozygous for Y_{11}, the catalase genes are silenced very efficiently (less than 3% total residual catalase activity), whereas in leaf protein extracts of plants hemizygous for Y_{11} reduction in catalase activity is less pronounced (between 13 and 59% total residual catalase activity). The relative high catalase activity in plants hemizygous for Y_{11} results from a less efficient silencing of both *CAT2* and *CAT3* transcripts, because quantitative real-time PCR analysis demonstrates that the level of *CAT3* mRNAs is reduced to the same extent as those of *CAT2*. However, the reduction at the RNA level is not as evident as that at the protein level, supporting previous data (Van Houdt *et al*., 1997) and implying that the silencing signal

can also act at the translational level. That *CAT3* with 75% identity to the primary target is transitively down-regulated is consistent with the observation that the expression of seven *RAC* gene family members in rice (*Oryza sativa*) was suppressed to variable degrees by a hairpin construct containing a highly conserved region of *OsRac1* or *OsRac5* (Miki *et al.*, 2005). On the other hand, the *CAT1* mRNA abundance is not significantly reduced in plants that are hemizygous, but somewhat in those homozygous for locus Y_{11}. This difference in susceptibility to silencing between *CAT1* and *CAT3* is most probably not due to a different degree of homology, because the sequence identity of the *CAT1* and *CAT3* genes with the Y_{11} transcript is very similar (78% and 75%, respectively) as is the number of stretches of at least 21 nt with full complementarity. A possible explanation is the different expression levels of the catalase genes: *CAT1* mRNAs are approximately 500 fold and 25 fold less abundant than *CAT2* and CAT3 transcripts, respectively. In plants hemizygous for Y_{11}, the reduction in *CAT2* and *CAT3* mRNAs is more pronounced than that in *CAT1* transcripts. However, homozygosity for locus Y_{11} would result in a double level of Y_{11} transcripts; consequently, more secondary siRNAs could increase the efficiency of targeting, thus inducing a strong reduction in the *CAT2* and *CAT3* mRNAs and some in the *CAT1* mRNA level. Kerschen *et al.* (2004) also observed that 25 different endogenous target sequences possess an inherent degree of susceptibility to RNA silencing induced by hairpin constructs. As they noted that genes with low expression levels could also become strongly silenced, they concluded that transcript accumulation is not the only target-specific determinant of silencing efficiency, but also that sequence composition, spatial and temporal gene expression patterns and the normal turnover rate of the targeted gene contribute to it (Kerschen *et al.*, 2004).

The positive correlation between the silencing efficiency and the zygosity of the primary target locus could be explained by a "threshold" model for transitive silencing, in which a certain level of primary transcripts has to be reached to activate the RDR6/Dicer-mediated pathway, in analogy to that proposed for silencing triggered by transgenes (De Neve *et al.*, 1999; De Wilde *et al.*, 2001; Elmayan and Vaucheret, 1996; Lindbo *et al.*, 1993; Que *et al.*, 1997; Schubert *et al.*, 2004; Tang *et al.*, 2003). However, plants hemizygous for locus Y_{11} do show transitive silencing, but less efficiently, suggesting that the amount of primary transcripts serving as template for RDR6 determines the level of secondary siRNAs produced, thus resulting in a certain degree of silencing of the secondary target. Analyses in a similar transitive XYZ system in tobacco have demonstrated that the accumulation level of secondary siRNAs depends on the zygosity of the target locus from which they are derived (García-Pérez *et al.*, 2004). Leaves of silenced XY rootstocks homozygous for locus Y produced approximately twofold the amount of secondary *gus*-specific siRNAs compared with that of rootstocks hemizygous for Y. In the *Arabidopsis* 5'-3' RNA exonuclease mutant *xrn4-1,* the efficiency of silencing has been shown to

depend on transgene dosage (Gazzani *et al.*, 2004). The mutated XRN4 protein was incapable of degrading its substrate, namely decapped RNAs originating from a transgene. The result was the accumulation of aberrant RNAs that could serve as the template for RDR6, leading to the suppression of the phenotype induced by the transgene. In plants that were hemizygous for the transgene, the phenotype was variable, while in plants homozygous for the transgene it was fully suppressed (Gazzani *et al.*, 2004).

The observation that the level of silencing in plants hemizygous for Y_{11} is to a lesser extent negatively correlated with the zygosity of locus X_{21} indicates that the primary silencing signal also plays a role. The presence of locus X_{21} in homozygous condition implies a larger population of primary siRNAs, which might result in a higher rate of RISC-mediated cleavage of Y_{11} templates. This hypothesis is supported by the fact that a hairpin construct controlled by the strong 35S promoter induces a stronger silencing phenotype than the same construct controlled by the weak nopaline synthase promoter (Chuang and Meyerowitz, 2000). In this case, the production of more hpRNAs and thus siRNAs leads to a more efficient degradation and silencing of the endogenous target RNAs. If this could be extrapolated, homozygosity of the silencing inducer X_{21} would result in a more efficient degradation of primary targets that are also the candidate template RNA molecules for RDR6. By consequence, a smaller population of secondary siRNAs would be produced leading to less efficient silencing of the endogenous target transcripts. García-Pérez *et al.* (2004) detected the same amount of secondary siRNAs in XY rootstocks homozygous for X compared to those hemizygous for X. However, the small decrease in silencing efficiency observed in plants homozygous for X_{21} compared to those hemizygous for X_{21} could result from only a minor reduction in siRNA production that cannot be discriminated on RNA gel blot. The observation that plants with both X_{21} and Y_{11} loci under homozygous condition are efficiently silenced suggests that the homozygosity of X_{21} is compensated by that of the Y_{11} locus, resulting in sufficient amounts of secondary siRNAs to down-regulate the *CAT2* and *CAT3* mRNAs and, to a lesser extent, those of *CAT1*.

García-Pérez *et al.* (2004) demonstrated a dosage dependency for the induction of systemic silencing via transitivity in tobacco. Grafting experiments with silenced rootstocks harbouring the silencing-inducing locus X and primary target locus Y indicated that rootstocks hemizygous for both loci were not able to produce a systemic silencing signal, while homozygosity of locus X and/or Y had a positive effect on the capacity of the rootstock to induce systemic silencing of a secondary target Z residing in the grafted scion. In contrast, we observe suppression in plants hemizygous for both X_{21} and Y_{11}, although less pronounced than in homozygous plants, with a negative influence of homozygosity of locus X_{21}, suggesting differences between transitive and systemic silencing signals.

In summary, we demonstrate that secondary siRNAs originating from a transgenic primary

target are able to down-regulate the expression of the endogenous catalase gene family in *A. thaliana*, resulting in a knock-down phenotype. Our experimental setup suggests that the expression of genes in gene families is determined by a subtle equilibrium between the gene expression level and the amount of siRNAs and could reflect an epigenetic gene regulation mechanism mediated by endogenous small RNAs.

Materials and Methods

Constructs

The T-DNA-derived plant transformation vector pDEchs287 (referred to as P35S-*npt*II-3'*chs*) carried a neomycin phosphotransferase II (*npt*II) gene (conferring kanamycin resistance) fused to the 3'-untranslated region (UTR) of the *Antirrhinum majus* chalcone synthase gene (3'*chs*, 287 nt) and transcribed from the cauliflower mosaic virus 35S promoter (P35S) towards the right T-DNA border. After floral dip transformation with the DEchs287 T-DNA, plants were obtained harbouring the IR locus X_{21} (see below; Fig. 1). The T-DNA vector pPs35SGUSnpt3'chs (referred to as P35S-*gus*-*npt*II-3'*chs*) contained two chimeric genes between the T-DNA borders: the bialaphos acetyltransferase-coding (*bar*) sequence conferring phosphinothricin resistance, fused to the 3'-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7 (3'*g7*) and under the control of the promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase (P*ss*) and a P35S-driven *gus* transgene fused to the last 122 nt of the *npt*II gene and the *Antirrhinum majus chs* terminator sequence (*npt*II-3'*chs*). After floral dip transformation with the Ps35SGUSnpt3'chs T-DNA, plants were obtained harbouring the single locus Y_7 (see below; Fig. 1). A high-throughput Gateway vector, pPs35SGW+npt3'chs, was constructed by inserting the reading frame A (rfA) sequence (1700 nt *Eco*RV fragment of the vector pGemGWrfA; Karimi *et al.*, 2002) into the vector pPs35Snpt3'chs (cut with *Pac*I and blunted with T_4-DNA polymerase). The orientation of the inserted *rfA* fragment was checked via sequencing. The T-DNA vector pPs35SGUS800npt3'chs (referred to as P35S-*gus*800-*npt*II-3'*chs*) was obtained through an LR clonase reaction between the vector pPs35SGW+npt3'chs and the entry clone pENTR3'GUS (obtained by a BP clonase reaction between pDONR™ and an *attB*-flanked PCR product containing the last 800 nt of the *gus*-coding sequence). In a similar manner, the T-DNA vector pPs35SCAT800npt3'chs (referred to as P35S-*CAT*800-*npt*II-3'*chs*) was constructed consisting of the last 800 nt of the *A. thaliana CAT2* sequence. After floral dip transformation with the Ps35SGUS800npt3'chs and Ps35SCAT800npt3'chs T-DNAs, plants harboured the single loci Y_9 and Y_{11} (see below; Fig. 1). The T-DNA H610 (H T-DNA in De Buck *et al.*, 1998, 2001) contained a *gus* gene fused to the 3'-UTR of the nopaline synthase gene (3'*nos*) under control of P35S and the hygromycin acetyl transferase (*hpt*) gene conferring resistance to hygromycin. Plants homozygous for the Z_c locus (Fig. 1) containing a single copy of the T-DNA H610, were already available. All the T-DNA vectors were inserted into the *Agrobacterium tumefaciens* strain C58C1RifR (pMP90).

Plant material, crosses and high-light treatment

All transformants were obtained via the floral dip procedure, performed as described by Clough and Bent (1998). Either wild-type *Arabidopsis thaliana* (L.) Heyhn. (ecotype Columbia) or transgenic plants homozygous for the Z_c

locus (--/--/Z_cZ_c plants) containing a single copy of the T-DNA H610, were used for the dipping experiments. Seeds of the dipped plants were harvested and sown on K1 medium supplemented with kanamycin (50 mg l^{-1}) for selection of the DEchs287 T-DNA, or phosphinothricin (10 mg l^{-1}) for selection of Ps35SGUSnpt3'chs, Ps35SGUS800npt3'chs and Ps35SCAT800npt3'chs. To obtain plants with a candidate X locus, --/--/Z_cZ_c plants were supertransformed with the DEchs287 T-DNA. Primary transformants (X-/--/Z_cZ_c) were retained with low amounts of the NPTII protein, indicative of silencing of the *npt*II gene(s) in the X locus, and with a high GUS activity, excluding transcriptional silencing of the *gus* gene in locus Z_c because of 35S-promoter homology. These plants were screened for harbouring a single locus and were further characterized through DNA gel blot analysis to determine the T-DNA integration pattern. Because the efficient *in trans*-silencing locus X in the tobacco XYZ system contained an IR about the right T-DNA border (Van Houdt *et al.*, 2003), a supertransformant with the T-DNAs in an IR configuration (locus X_{21}, Fig. 1), namely X_{21}-/--/Z_cZ_c, was chosen for further handling. To obtain X_{21}-/--/-- plants, seeds from a self-fertilized X_{21}-/--/Z_c- plants (obtained after crossing X_{21}-/--/Z_cZ_c with a --/Y-/-- plant, not discussed here) were sown on K1 medium supplemented with kanamycin, and the presence of locus Z_c was checked through a callus induction test on hygromycin-containing M1 (20 mg l^{-1}) medium. Only one plant out of 52 kanamycin-resistant plants was hygromycin sensitive, suggesting that locus X_{21} and locus Z_c were genetically linked. To obtain candidate Y plants, --/--/Z_cZ_c plants were supertransformed with the Ps35SGUSnpt3'chs and Ps35SGUS800npt3'chs T-DNAs, and wild-type plants were dipped with *Agrobacterium tumefaciens* containing the T-DNA Ps35SCAT800npt3'chs. Fluorometric GUS assays and catalase activity assays were performed to select primary transformants with highly expressed Y transgenes, excluding silencing of the *gus* transgene of Z_c or the endogenous catalase genes. Via segregation analysis we ensured the presence of single loci, leading to the selection of transformants --/Y_7-/Z_cZ_c (Ps35SGUSnpt3'chs), --/Y_9-/Z_cZ_c (Ps35SGUS800npt3'chs) and --/Y_{11}-/-- (Ps35SCAT800npt3'chs). Transitive silencing was studied by crossing progeny plants of X_{21}-/--/Z_cZ_c with those of --/Y_7-/Z_cZ_c, --/Y_9-/Z_cZ_c and --/Y_{11}-/--, and the resulting hybrid seeds were grown on medium selective for the presence of X_{21} and Y_7, Y_9 or Y_{11}. After 2 weeks, all plants were transferred to soil and grown under a light regime of 16 h day/8 h night, at 21°C. For high-light (HL) treatment, 4-week-old plants were exposed to continuous HL irradiation (approximately 1000 µmol photons m^{-2} sec^{-1}).

NPTII and GUS quantification

Six weeks after sowing on selective medium, two rosette leaves were harvested, frozen in liquid nitrogen, and ground in 200 µl extraction buffer (0.1 M phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2-EDTA, and 0.1% Triton X-100). The homogenate was centrifuged (15,000x*g*) twice at 4°C for 10 min to remove cell debris. The extracts were stored in 25% (v:v) glycerol at -70°C. The protein concentration of the extracts was determined with the BioRad Protein Assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The NPTII ELISA was done according to the manufacturer's instructions (Pathoscreen Kit for Neomycin Phosphotransferase II; Agdia, Elkhart, IN, USA). The microtitre plates were read immediately after adding the TMB peroxidase substrate solution, at 405 nm and at 37°C, with a kinetic program (SOFTMaxPRO; Molecular Devices, Sunnyvale, CA, USA) with 2 min intervals for 40 min. GUS activity, expressed as units of GUS protein relative to the total amount of soluble extracted protein (U GUS mg^{-1} TSP) was determined as described by Breyne *et al.* (1993).

DNA gel blot analysis

Genomic DNA from leaf tissue was isolated with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). DNA gel blot analysis was done as described previously (Van Houdt *et al.*, 2003).

Catalase activity assay

Protein extracts were prepared from two rosette leaves, frozen in liquid nitrogen, by grinding in 120 µl extraction buffer (60 mM Tris-HCl (pH 6.9), 1 mM phenylmethylsulfornylfluoride, 10 mM DTT, 20% glycerol). The homogenate was centrifuged (15,000x*g*) twice at 4°C for 10 min to remove insoluble material. The supernatant was used for spectrophotometric catalase analysis according to Clare *et al.* (1984) after determining the total amount of soluble protein with the BioRad Protein Assay (Bradford, 1976) with BSA as a standard.

Quantitative real-time PCR analysis

For the quantification of *CAT1*, *CAT2* and *CAT3* transcript levels by real-time PCR, total RNA from leaf material of 5-week-old plants was prepared with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 1 µg of DNase-treated total RNA was reverse-transcribed with the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) according to the instruction manual. First-strand cDNA was generated with an oligo$(dT)_{12\text{-}18}$ primer. Of the first-strand cDNA, 0.2 µl was used as a template in a subsequent qPCR using the qPCRTM Core Kit for SYBR® Green I (Eurogentec, Seraing, Belgium). The transcripts were amplified with gene-specific primers: 5'-TGCTCCTCTTACGGTTTGGTTTC-3' and 5'-ACCCTTCTTTAAGCGTTTCATTGTC-3' for *CAT1*; 5'-GCTGGCAAGCCGTCTGAAC-3' and 5'-AGCACAGAAGATCCACATGATGAAG-3' for *CAT2*; 5'-GGCCAATCTCCATATAAGCTCAGT-3' and 5'-GGATTTAACGACCAAGCGATGATAG-3' for *CAT3*; 5'-AACTTGTGCTCATCTGCCATTAGG-3' and 5'-TGATTCTGCGGAAACACCACTTTAG-3' for *actin2* (At3g60830).

Small RNA analysis

Small RNAs were detected as described by Van Houdt *et al*. (2003), with minor changes. Low molecular weight RNA (35 µg) was loaded on the gel, as well as 21 and 24 nt RNA oligomers as size controls. The probe, a PCR fragment of 274 bp amplified with the primers 5'-CCGGTTAATTAAATGCTGAGAAGTATCCAACT-3' and 5'-CCGGTTAATTAACGGCTTGCCAGCTTCTGTCC-3', was labelled with [α-^{32}P]dCTP using the Rediprime™ II random prime labelling system (GE-Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. After removal of unincorporated [α-^{32}P]dCTPs with the Bio-Spin p30 Column (Bio-Rad Laboratories, Hercules, CA, USA), the probe was hydrolyzed into fragments of approximately 50 nucleotides. Hybridization and washes were performed as described at 50°C. Labelled membranes were exposed to a phosphorImager screen (GE-Healthcare). Predominantly ethidium bromide-stained species of low molecular weight RNA, separated by agarose gel electrophoresis (1 µg per lane) were used as loading controls.

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Chapter 5:

Factors influencing the efficiency of transitive silencing

Submitted for publication:

Bleys, A., Vermeersch, L., Van Houdt, H., and Depicker, A. (2006) The efficiency of endogene suppression by transitive silencing signals is influenced by the length of sequence homology between primary and secondary targets.

Factors influencing the efficiency of transitive silencing

Abstract

Transitivity, the spread of RNA silencing along primary target sequences, leads to the degradation of secondary targets without sequence homology to the initial silencing trigger. We demonstrate that increasing the distance between directly targeted and adjacent sequences in a transgenic primary target delays the onset of silencing of a secondary target gene. Silencing can spread in 3' to 5' direction over a distance of at least 500 nt, but this appears to require more time compared to a distance of 98 nt or 250 nt. The efficiency and frequency of transitive silencing of an endogene depends on the length of its sequence homology with the primary target. With a length of 500 nt, efficient silencing can eventually be established in all plants, whereas lengths of 250 nt and 98 nt homology result in less efficient and less frequent suppression. These results suggest that amplification of secondary siRNAs is a time-requiring process that gradually expands the population of siRNAs until a steady-state level is reached. Moreover, the length of the primary target providing secondary siRNAs determines whether this steady-state level readily exceeds the threshold necessary for efficient silencing.

Introduction

A wide variety of eukaryotic organisms, including plants, animals and fungi, have developed several RNA silencing pathways to protect their cells and genomes against invading nucleic acids, such as viruses or transposons. The RNA silencing pathways also regulate gene expression during development or in response to external stimuli (for recent reviews, see Baulcombe, 2005; Meins *et al.*, 2005). All RNA silencing systems involve the processing of dsRNA into smRNAs of 21-25 nt by an RNaseIII-like enzyme, known as Dicer or Dicer-like (Bernstein *et al.*, 2001; Xie *et al.*, 2004, 2005; Dunoyer *et al.*, 2005). These smRNAs are incorporated into silencing effector complexes containing an AGO protein (for review, see Meister and Tuschl, 2004). Variations on this core mechanism include different origins of the silencing trigger and different types of effector complexes. Endogenous, viral or transgenic RNA molecules can give rise to dsRNA by fold-back of IR sequences, by hybridization of sense and antisense sequences, or by the action of an RNA-dependent RNA polymerase (RDR). Cleavage of the dsRNAs leads to the formation of transgenic and viral siRNAs, endogenous microRNAs, *trans*-acting siRNAs or chromatin-associated siRNAs. Depending on the dsRNA source, the smRNAs are recruited into different effector complexes (RISC or RITS complex), resulting in sequence-specific RNA degradation, translational repression or chromatin modifications (Béclin *et al.*, 2002; Zilberman *et al.*, 2003; Baumberger and Baulcombe, 2005).

Although the basic mechanism for RNA silencing is evolutionarily conserved, only nematodes, fungi and plants have developed an RDR-dependent amplification system ensuring a robust RNA silencing response that can expand and spread in the organism (Sijen *et al.*, 2001; Martens *et al.*, 2002; Forrest *et al.*, 2004). Amplification involves the production of dsRNA by the action of an RDR, using primary target RNAs as template. The newly synthesized dsRNA is subsequently cleaved into siRNAs that are able to guide the degradation of additional secondary target RNAs. This mechanism also enables cells to respond to silencing signals that travel throughout the organism, resulting in systemic silencing (Klahre *et al.*, 2002; Alder *et al.*, 2003; Himber *et al.*, 2003; García-Pérez *et al.*, 2004; Schwach *et al.*, 2005). A further characteristic of RDR-mediated amplification is the production of secondary siRNAs from regions outside of the sequence initially targeted by trigger-derived primary siRNAs, termed transitivity (Sijen *et al.*, 2001). Transitive silencing has first been described in *Caenorhabditis elegans*, where it proceeds over a distance of a few hundred nucleotides in the 3' to 5' direction only, through dsRNA synthesis primed by primary siRNAs (Sijen *et al.*, 2001). In this organism, spreading of silencing is not limited to transgenic targets, but also endogenous transcripts can be used as template for amplification (Sijen *et al.*, 2001; Alder *et al.*, 2003). In plants, transitive silencing can occur in both the 3' to 5' and 5' to 3' direction along transgenic target RNAs in a primer-dependent or primer-independent manner (Braunstein *et al.*, 2002; Vaistij *et al.*, 2002; Himber *et al.*, 2003; Van

Houdt *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005). Virus-induced gene silencing (VIGS) has been shown to spread over a distance of at least 1000 nt from the 5' end to the 3' end of the target mRNA, while 3' to 5' spreading can extend at least through 332 nt, with a possible limit of 600 nt (Vaistij *et al.*, 2002; Petersen and Albrechtsen, 2005). In plants, many studies failed to demonstrate transitivity along endogenous transcripts, suggesting that endogenous sequences are protected from transitivity by some inherent feature (Vaistij *et al.*, 2002; Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005). Indeed, only one report showed the involvement of endogenous transcripts in signal amplification and selection of homologous targets (Sanders *et al.*, 2002).

In an XYZ system in tobacco we have shown that transitive amplification products that arise from silenced targets containing transgenic β-glucuronidase (*gus*) sequences are able to trigger silencing of an additional *gus* transgene (Van Houdt *et al.*, 2003; Chapter 3). We have now implemented this system in *Arabidopsis thaliana* and showed that both transgenes and endogenes can be silenced by the secondary transitive signals (Chapter 4). In this XYZ system, the primary target Y harbors a direct target region homologous to the silencing inducer X and an adjacent region homologous to the secondary target Z. Here, we investigated the effect on the timing of transitive silencing of increasing the distance in the primary target between the homologies with the silencing inducer and the secondary target. We also analyzed the influence on the frequency and efficiency of transitive silencing of increased length of sequence homology between primary and secondary targets. We used three primary target Y constructs in which different lengths of the catalase 2 (*CAT2*) sequence were inserted between the 3' end targeted by the silencing inducer X and the upstream region homologous to a *gus* target Z. We assessed the occurrence of transitive silencing of both secondary targets *CAT* and *gus*, looked at the relationship between silencing efficiency and secondary siRNA production, and checked the accumulation of tertiary endogenous siRNAs.

Results

Increasing the distance between directly targeted and adjacent sequences in a transgenic primary target delays the onset of transitive gus silencing

In tobacco, a post-transcriptionally silenced IR of a T-DNA with a highly transcribed transgene, designated as locus X, can trigger transitive silencing of a secondary target Z when a stepwise homology is created through the introduction of a chimeric primary target Y with homology to both the silencing inducer X and the secondary target Z (Van Houdt *et al.*, 2003; Chapter 3). Similarly, in *Arabidopsis*, locus X_{21} (Fig. 1; IR of P35S-*npt*II-3'*chs*) can transmit its silencing-inducing

capacity to the primary target Y_7 (Fig. 1; P35S-*gus*-*npt*II3'*chs*), which in turn is able to silence the transgenic *gus* target Z_c (Fig. 1; P35S-*gus*-3'*nos*; Chapter 4). To assess whether the frequency of transitive *gus* silencing varies with an increasing distance between the direct target and adjacent sequences located in Y, we constructed three primary targets Y_{98}, Y_{250} and Y_{500}. For this purpose, we used construct Y_7, in which we inserted 98 nt, 250 nt and 500 nt of the *CAT2*-coding sequence between the *npt*II-3'*chs* region initially targeted by the silencing trigger X_{21}, and the full-length *gus*-coding sequence homologous to the secondary target Z_c. Plants harboring different combinations of locus X_{21}, one of the four single-copy loci Y (Y_7, Y_{98}, Y_{250} or Y_{500}) and Z_c, were obtained after floral dip transformation, crossing experiments and self-fertilization (see "Materials and Methods").

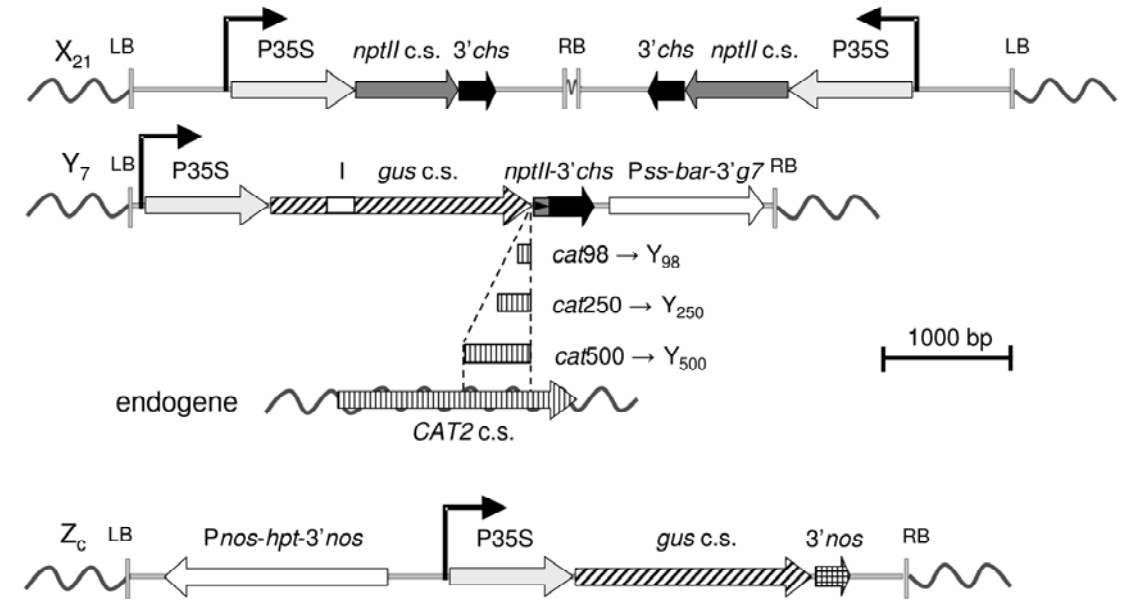
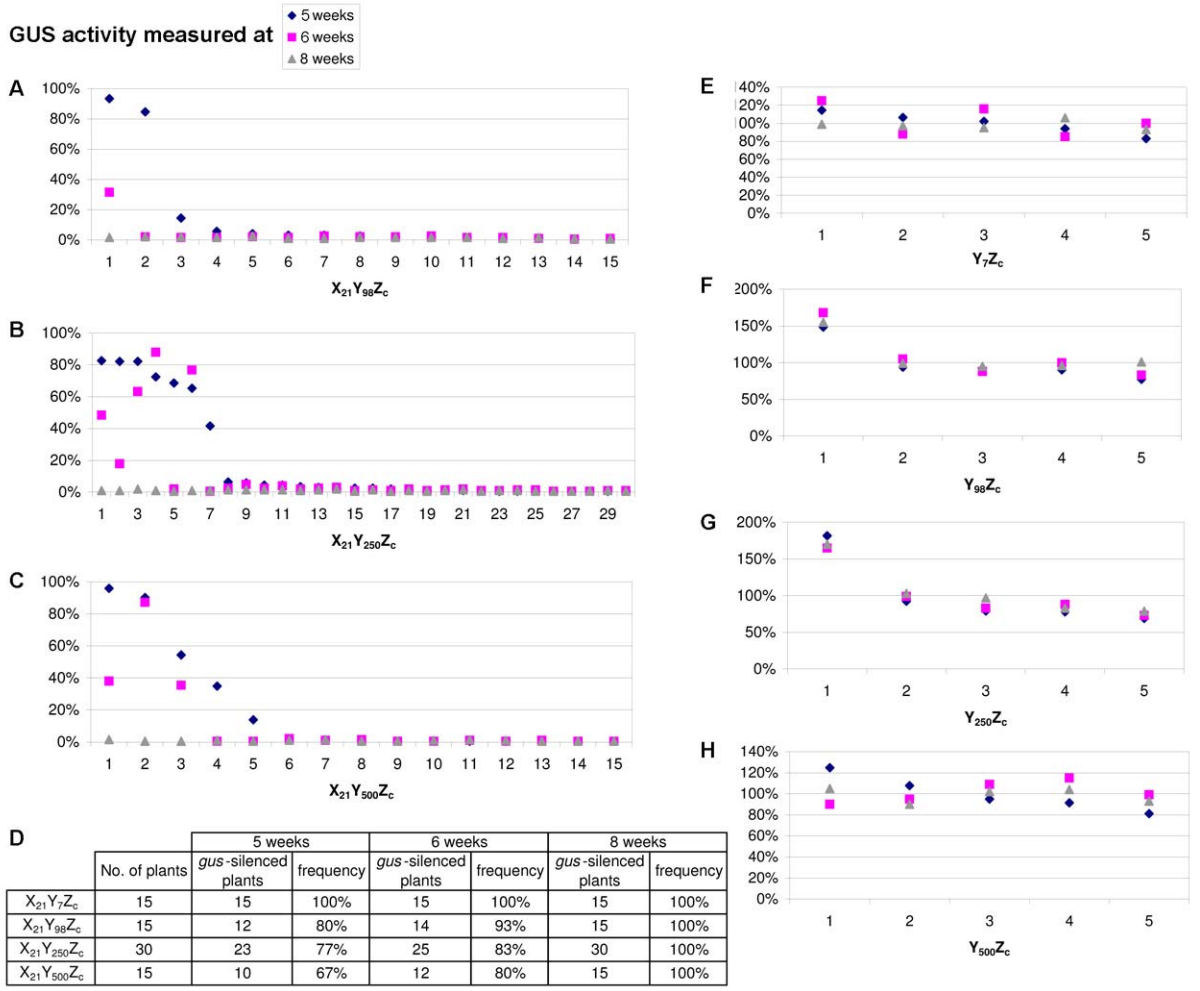


Figure 1. Schematic outline of the T-DNA constructs (drawn to scale), present in locus X_{21}, primary targets Y_7, Y_{98}, Y_{250} and Y_{500}, and secondary target Z_c. The silencing-inducing locus X_{21} contains two T-DNA copies arranged as an IR around the right border. The Y_{98}, Y_{250} and Y_{500} loci and locus Z_c are single-copy loci. The sequences that are inserted into Y_7 between the *npt*II-*t*3'*chs* region targeted by the silencing trigger X_{21} and the full-length *gus*-coding sequence (*gus* c.s.) homologous to secondary target Z_c, are homologous to the 3' part of the *Arabidopsis CAT2*-coding sequence (*CAT2* c.s.). As indicated on the scheme, the inserts overlap each other and are 98 nt, 250 nt and 500 nt in length (*cat*98, *cat*250 and *cat*500), giving rise to Y_{98}, Y_{250} and Y_{500}. The curled lines represent plant DNA. 3'*chs*, 3'-UTR of the chalcone synthase gene of *Anthirrinum majus*; 3'g7, 3'-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7; 3'*nos*, 3'-UTR of the nopaline synthase gene; *bar*, bialaphos acetyltransferase-coding sequence conferring phosphinothricin resistance; *hpt*, hygromycin phosphotransferase-coding sequence; I, artificial intron; *npt*II c.s., neomycin phosphotransferase II-coding sequence; P35S, CaMV 35S promoter; P*nos*, nopaline synthase promoter; P*ss*, promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase; LB, left T-DNA border; RB, right T-DNA border.

The GUS activity was measured in leaf protein extracts from $X_{21}YZ_c$ and YZ_c plants for each Y locus, at three different time points (Fig. 2). Because all YZ_c plants (5 plants for every Y construct) had a high GUS activity (Fig. 2E, F, G and H), we can conclude that none of the Y constructs induces *in cis* or *in trans* silencing of the *gus* genes in the absence of locus X_{21}. The criterion for discriminating silenced from non-silenced $X_{21}YZ_c$ plants was a GUS activity value that was less than 10% of the average GUS activity of the corresponding YZ_c plants. Figure 2 shows the GUS activity of $X_{21}Y_{98}Z_c$ (A), $X_{21}Y_{250}Z_c$ (B) and $X_{21}Y_{500}Z_c$ (C) plants measured at 5, 6 and 8 weeks after sowing. On the basis of this criterion, all 15 $X_{21}Y_7Z_c$ plants exhibited very efficient *gus* silencing that was already established at 5 weeks (Fig. 2, D). This result is in contrast to those obtained for 15 $X_{21}Y_{98}Z_c$, 30 $X_{21}Y_{250}Z_c$ and 15 $X_{21}Y_{500}Z_c$ plants (Fig. 2A, B, and C, respectively), which at 5 weeks had a variable frequency of silencing (80%, 77% and 67%, respectively). Although these frequencies do not differ strongly from each other, it seems that the frequency of *gus* silencing decreased as a function of the length of the *CAT2* insert. After 6 weeks, more plants became silenced (93%, 83% and 80%, respectively), and after 8 weeks, all plants had suppressed the *gus* genes. In plants that were not silenced based on the criterion, the GUS activity ranged from 14% to 96% of the average GUS activity of the corresponding YZ_c plants (Fig. 2A, B, and C). The occurrence of different degrees of silencing (referred to as silencing efficiency) indicates that transitive silencing was induced gradually or that in some plant cells silencing was complete, while in others it was not established yet. From these data, we can conclude that the onset of silencing of Z_c, induced by a transitive silencing signal produced by the primary target Y, is delayed when the upstream *gus* sequence in Y is separated by only 98 nt from the 3' end region targeted by the X_{21}-derived primary siRNAs. The timing of silencing seemed to be negatively correlated with the distance between direct target and adjacent sequences.



	No. of plants	5 weeks		6 weeks		8 weeks	
		gus-silenced plants	frequency	*gus*-silenced plants	frequency	*gus*-silenced plants	frequency
$X_{21}Y_7Z_c$	15	15	100%	15	100%	15	100%
$X_{21}Y_{98}Z_c$	15	12	80%	14	93%	15	100%
$X_{21}Y_{250}Z_c$	30	23	77%	25	83%	30	100%
$X_{21}Y_{500}Z_c$	15	10	67%	12	80%	15	100%

Figure 2. GUS activity measured in $X_{21}Y_{98}Z_c$ (A), $X_{21}Y_{250}Z_c$ (B) $X_{21}Y_{500}Z_c$ (C), Y_7Z_c (E), $Y_{98}Z_c$ (F), $Y_{250}Z_c$ (G), and $Y_{500}Z_c$ (H) plants, measured in protein extracts made of leaf tissue harvested at 5 weeks, 6 weeks and 8 weeks. The GUS activity of the individual plants is represented as the percentage of the average GUS activity value of the corresponding YZ_c plants. The results are ranked based on decreasing GUS activities at 5 weeks. In D, the number of plants analyzed, the number of silenced $X_{21}YZ_c$ plants (with a GUS activity value less than 10% of the average GUS activity of the corresponding YZ_c plants) and the frequency of *gus* silencing is shown.

The efficiency of gus silencing is correlated with the amount of secondary siRNAs

The delay in *gus* silencing observed in some $X_{21}YZ_c$ plants suggests that spreading of silencing along the Y transcripts proceeds gradually through the production of an increasing amount of secondary *gus*-specific siRNAs. Initially, amplification would result in a small amount of secondary siRNAs that is insufficient to induce strong suppression, but eventually, enough *gus* siRNAs would be produced leading to efficient transitive silencing of Z_c. To test this hypothesis, we evaluated the accumulation of siRNAs in non-silenced YZ_c plants as a control and in $X_{21}YZ_c$ plants that were efficiently, intermediately or not silenced. An RNA gel blot using a hydrolyzed

^{32}P-labeled probe comprising the full-length *gus*-coding sequence revealed 21-nt long *gus*-specific siRNAs in the low-M_r RNA fraction of $X_{21}YZ_c$ plants that showed efficient (Fig. 3A, lanes 7 to 10) or intermediate silencing (lanes 5 and 6), whereas non-silenced $X_{21}Y_{98}Z_c$ (lane 4) and YZ_c plants (lanes 1 to 3) did not accumulate *gus* siRNAs. The signal intensities in intermediately silenced $X_{21}YZ_c$ plants were clearly weaker than those in plants that had low GUS activities. Thus, there seems to be a correlation between the amount of secondary *gus* siRNAs produced and the reduction in GUS activity. Whether the intermediate levels are the result of overall intermediate siRNA accumulation or the sum of high levels of siRNAs in silenced and low levels in non-silenced cells is not known.

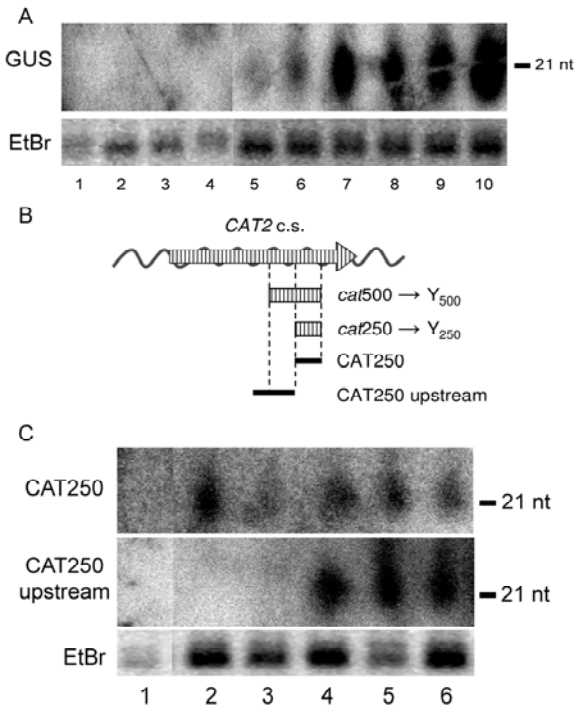
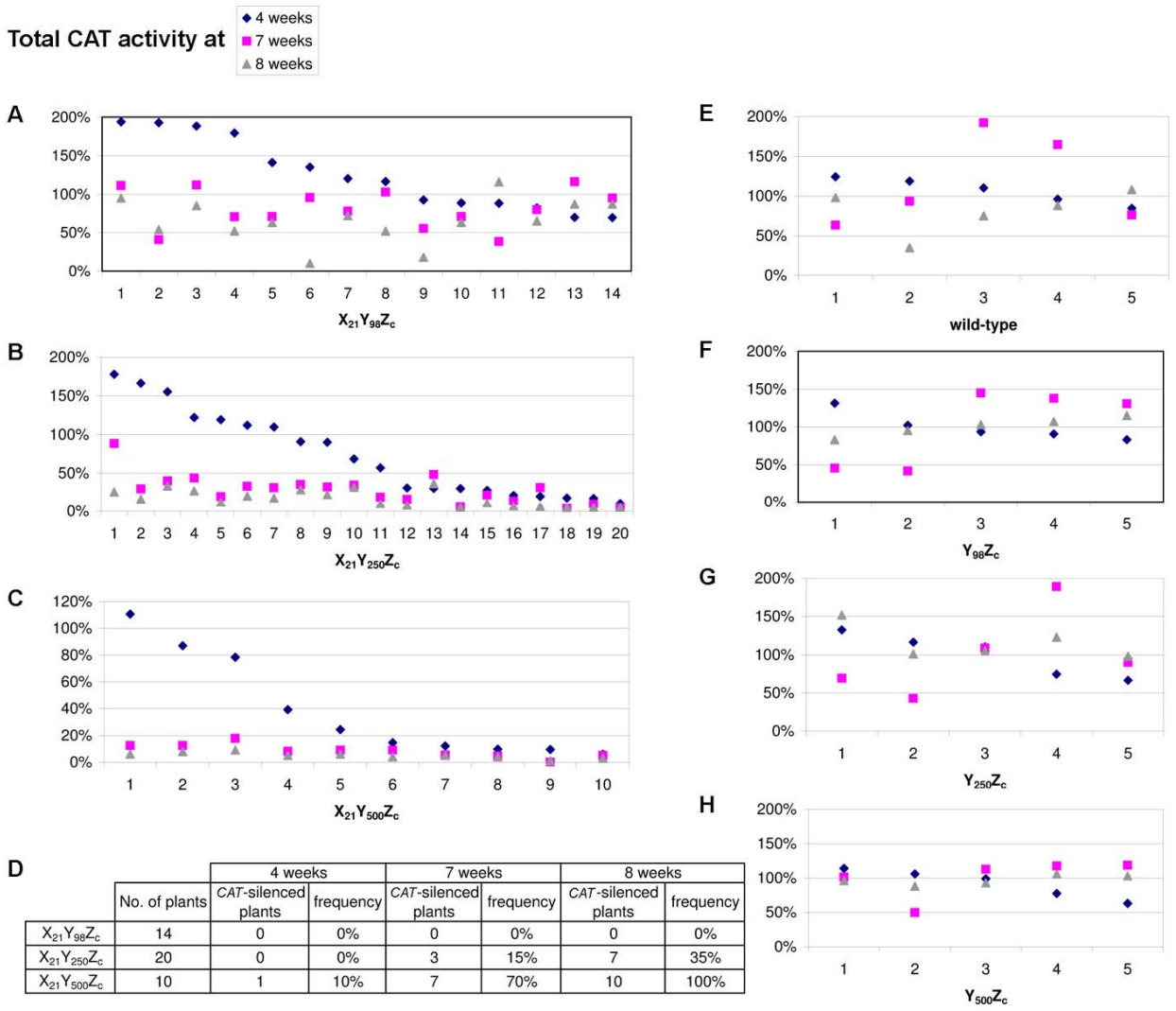


Figure 3. Accumulation of siRNAs. RNA oligomers of 21 nt were used as molecular marker. The predominant ethidium bromide (EtBr)-stained species in the low-M_r RNA fraction are shown as loading controls. **(A)** Detection of *gus* siRNAs. Each numbered lane contains the low-M_r RNA fraction extracted from leaves of individual plants at different time points. siRNAs were detected with a hydrolyzed ^{32}P-labeled probe comprising the full-length *gus*-coding sequence. Lane 1, $Y_{98}Z_c$; lane 2, $Y_{250}Z_c$; lane 3, $Y_{500}Z_c$; lane 4, $X_{21}Y_{98}Z_c$ not silenced for *gus* (98%); lane 5, $X_{21}Y_{250}Z_c$ with intermediate GUS activity (36%); lane 6, $X_{21}Y_{500}Z_c$ with intermediate GUS activity (36%); lane 7, *gus*-silenced $X_{21}Y_{98}Z_c$; lane 8 and 9, *gus*-silenced $X_{21}Y_{250}Z_c$; lane 10, *gus*-silenced $X_{21}Y_{500}Z_c$. **(B)** Schematic illustration of the position of the probes used to detect *CAT2* siRNAs (drawn to scale). The probe 'CAT250' corresponds to the *CAT2* insert (*cat*250) present in Y_{250}; the probe 'CAT250 upstream' covers 398 nt of the *CAT2* sequence located upstream of *cat*250 and partially present in Y_{500} (*cat*500). **(C)** Detection of *CAT2* siRNAs. Each numbered lane contains the low-M_r RNA fraction extracted from leaves of individual 7-week-old plants. The upper panel shows the siRNAs detected with the 'CAT250' probe, the lower with 'CAT250 upstream'. Lane 1, $Y_{250}Z_c$; lane 2 and 3, *CAT* silenced $X_{21}Y_{250}Z_c$; lane 4, 5 and 6, *CAT* silenced $X_{21}Y_{500}Z_c$.

Increasing the length of sequence homology between primary and secondary target increases the frequency and efficiency of transitive CAT2 silencing

With the same constructs and plants, we checked whether the length of the *CAT2* inserts was correlated with the frequency and efficiency of *CAT* silencing. The *Arabidopsis CAT2* gene targeted by the Y_{98}, Y_{250} and Y_{500} constructs is part of a gene family (*CAT1*, *CAT2* and *CAT3*), with a nucleotide and amino acid sequence identity of 70-72% and 75-84%, respectively (Frugoli *et al.*, 1996). The *CAT2* and *CAT3* genes are highly expressed in leaves. With the CAT activity assay (see "Materials and Methods"), the total CAT activity was determined in protein extracts from leaves of 4-, 7- and 8-week-old $X_{21}YZ_c$ and YZ_c plants. All YZ_c plants (5 plants for every Y construct) had variable CAT activities that were comparable to those of wild-type plants (Fig. 4E, F, G and H), so we can conclude that none of the Y constructs induces *in cis* or *in trans* silencing of *CAT* in the absence of locus X_{21}. Then we determined the total CAT activity in $X_{21}YZ_c$ plants. The criterion for discriminating silenced from non-silenced $X_{21}YZ_c$ plants was a CAT activity value that was less than 10% of the average CAT activity of the corresponding YZ_c plants. Figure 4 shows the total CAT activity of $X_{21}Y_{98}Z_c$ (A), $X_{21}Y_{250}Z_c$ (B) and $X_{21}Y_{500}Z_c$ (C) plants measured at 4, 7 and 8 weeks after sowing. Nine out of ten 4-week-old $X_{21}Y_{500}Z_c$ plants (Fig. 4D) exhibited a delay in the onset of *CAT* silencing, with CAT activities ranging from 10% to 102% of the average CAT activity of the $Y_{500}Z_c$ plants (Fig. 4C). After 7 weeks, three plants were not silenced based on this same criterion, but those plants had CAT activities of 12 to 18%, and were silenced after 8 weeks. In contrast, none of the 20 $X_{21}Y_{250}Z_c$ plants showed silencing after 4 weeks (Fig. 4D), with CAT activities ranging from 11% to 160% (Fig. 4B). The percentage of silenced plants increased to 15% after 7 weeks, but after 8 weeks only seven out of 20 plants strongly suppressed *CAT*, although all the other plants had intermediate CAT activities, ranging from 10% to 37% of the average CAT activity of the $Y_{250}Z_c$ plants. None of the 14 $X_{21}Y_{98}Z_c$ plants (Fig. 4D) showed a strong reduction in total CAT activity after 4 and 7 weeks, with the CAT activities ranging from 50 to 165%, and from 37 to 111%, respectively (Fig. 4A). After 8 weeks, none of the plants were silenced based on the criterion, but two plants had a CAT activity of 10 and 18%, while the other 13 plants showed a total CAT activity of 52 to 116% (Fig. 4A). We can conclude that only with primary target Y_{500} a silencing frequency of 100% could be obtained after 8 weeks, whereas with Y_{250} and Y_{98} not all $X_{21}YZ_c$ plants reached strong *CAT* suppression. At this time point, the frequency and efficiency of silencing in $X_{21}Y_{250}Z_c$ plants was higher compared to $X_{21}Y_{98}Z_c$ plants. Thus, the extent of homology between primary and secondary target did significantly influence the degree of silencing and the probability of obtaining strong suppression.



	No. of plants	4 weeks		7 weeks		8 weeks	
		CAT-silenced plants	frequency	*CAT*-silenced plants	frequency	*CAT*-silenced plants	frequency
$X_{21}Y_{98}Z_c$	14	0	0%	0	0%	0	0%
$X_{21}Y_{250}Z_c$	20	0	0%	3	15%	7	35%
$X_{21}Y_{500}Z_c$	10	1	10%	7	70%	10	100%

Figure 4. Total CAT activity measured in $X_{21}Y_{98}Z_c$ (A), $X_{21}Y_{250}Z_c$ (B) $X_{21}Y_{500}Z_c$ (C), wild-type (E), $Y_{98}Z_c$ (F), $Y_{250}Z_c$ (G), and $Y_{500}Z_c$ (H) plants, measured in protein extracts made of leaf tissue harvested at 4 weeks, 7 weeks and 8 weeks. The total CAT activity of the individual plants is represented as the percentage of the average CAT activity value of the corresponding YZ_c plants. The results are ranked based on decreasing CAT activities at 4 weeks. In D, the number of plants analyzed, the number of *CAT*-silenced $X_{21}YZ_c$ plants (with a CAT activity value less than 10% of the average CAT activity of the corresponding YZ_c plants) and the frequency of *CAT* silencing is shown.

A transitively silenced catalase gene does not give rise to tertiary siRNAs

To assess whether endogenous transcripts that are targeted by secondary siRNAs originating from a primary target Y in turn produce tertiary siRNAs, we characterized the siRNA population in $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants that showed efficient *CAT* silencing and in a non-silenced $Y_{250}Z_c$ plant. First, we used the hydrolyzed ^{32}P-labeled probe 'CAT250' corresponding to the

CAT2 insert present in Y_{250} (Fig. 3B), revealing that all $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants produced secondary *CAT2*-specific siRNAs (Fig. 3C, upper panel, lanes 2 to 6), whereas in the $Y_{250}Z_c$ plant no siRNAs were detected (lane 1). Because it is not possible to discriminate between on the one hand secondary siRNAs originating from primary target Y and on the other hand tertiary siRNAs produced by endogenous *CAT* transcripts with this probe, we stripped the membrane and re-hybridized it with the hydrolyzed riboprobe 'CAT250 upstream' (Fig. 3B). With this probe we could detect secondary siRNAs originating from Y_{500} in $X_{21}Y_{500}Z_c$ plants (Fig. 3C, lower panel, lanes 5 and 6), whereas in the $X_{21}Y_{250}Z_c$ plants no signal was detectable. This observation suggests that endogenous CAT transcripts do not participate in the amplification of tertiary siRNAs, or that the endogenous mRNA-derived siRNAs were produced below detection level.

Discussion

Here, we study in more detail the 3' to 5' spreading previously observed with the XYZ-silencing system (Van Houdt *et al.*, 2003; Chapter 4) by using primary targets Y_{98}, Y_{250} and Y_{500} in which 98 nt, 250 nt and 500 nt of the *CAT2* sequence, respectively, were inserted between the *npt*II-3'*chs* end targeted by the silencing inducer X_{21} and the upstream region homologous to a transgenic *gus* target Z_c. We demonstrate that the length of the *CAT2* inserts influences the timing and efficiency of silencing induced by secondary *CAT2*- and *gus*-specific siRNAs originating from the primary target transcripts. Increasing the distance between primary and secondary target regions in Y by larger *CAT2* inserts delays the onset of transitive *gus* silencing, whereas increasing the length of sequence homology with the endogenous *CAT2* target increases the frequency and efficiency of transitive *CAT* silencing.

According to the current model for transitive silencing in *Arabidopsis*, transcripts that are targeted by trigger-derived siRNAs can in some way be recognized by RDR6 as templates for the production of dsRNA (Vaistij *et al.*, 2002; Himber *et al.*, 2003; for review, see Bleys *et al.*, 2006). These new dsRNAs are subsequently processed into secondary siRNAs that can target homologous transcripts without sequence identity to the initial silencing inducer. Our findings suggest that transitivity requires time to build up a certain steady-state level of secondary siRNAs that results in a corresponding maximum plateau level of silencing. Similarly, it has been shown in tobacco plants homozygous for a T-DNA locus containing (a) sense *Nicotiana plumbaginifolia* β-1.3-glucanse *gn1* gene construct(s) that the suppression of *gn1* gene expression increased gradually after 4 weeks (de Carvalho *et al.*, 1992). The structure of this locus is not known, but it might harbor an IR combined with an extra target locus.

An increasing ratio of stochastically silenced to non-silenced cells could account for the observed transition from no or less strong transitive silencing to an efficient silencing response through time (Figures 2 and 4). However, because transitive silencing is not a cell autonomous process (Himber *et al.*, 2003; García-Pérez *et al.*, 2004), it is likely that a general secondary siRNA level is produced that can induce some degree of suppression. Indeed, we demonstrated that the efficiency of suppression of catalase expression by secondary siRNAs depends on the zygosity of the primary target locus (Chapter 4). Plants hemizygous for locus X_{21} and locus Y_{11}, containing only the last 800 bp of the *CAT2* sequence upstream of the *npt*II-3'*chs* end, showed at 4 weeks intermediate silencing efficiencies, ranging from 13 to 34% of the average catalase activity in wild-type *Arabidopsis*. However, plants homozygous for locus Y_{11} exhibited very efficient catalase silencing. This suggested that the amount of primary transcripts serving as template for RDR6 determines the level of secondary siRNAs produced, resulting in a certain degree of silencing of the secondary target. The efficiencies after 4 and 6 weeks were very similar (data not shown), which is in contrast to the temporal increase in the efficiency and frequency of catalase silencing we describe in this Chapter. It could be that the transcripts originating from the Y_{98}, Y_{250} and Y_{500} loci are more stable than those of Y_{11}, because they are not truncated, but encode a functional GUS protein. The higher levels of RNAs that can act as template for RDR6 would gradually give rise to a higher steady-state level of secondary siRNAs that results in silencing with increasing efficiency. Depending on whether this steady-state level exceeds a certain threshold, very efficient silencing can be obtained. Our observation that not only the length of sequence homology between primary and secondary targets (800 bp in Y_{11} versus 500 bp Y_{500}), but possibly also the stability of Y transcripts influences the silencing response, suggests that the limiting factor for dsRNA synthesis is the availability of substrates for RDR6. This is consistent with the observation that the activation and maintenance of PTGS, termed 'quelling' in the fungus Neurospora crassa, appear to rely on the amount of both the RDR QDE-1 and its transgenic RNA substrates (Forrest *et al.*, 2004). Overexpression of QDE-1 resulted in a dramatic increase in the frequency of silencing, coinciding with an increase in the quantity of siRNAs. Accordingly, we observe a positive correlation between the amount of secondary siRNAs and the level of suppression. Accordingly, we observed a positive correlation between the amount of secondary siRNAs and the level of suppression (Fig. 3A).

Assuming that based on a certain amount of template a corresponding number of secondary siRNAs can be made, increasing the length of substrate results in a larger population of siRNAs and a concomitant increased degradation of the secondary target. Indeed, we observed that the length of sequence homology between primary and secondary targets strongly influences the silencing response (Fig. 4). With a length of 500 nt, efficient *CAT* silencing can eventually be established in all plants, whereas a length of 250 nt homology

results in less frequent and less efficient suppression and a length of 98 nt homology has nearly no effect.

Because primary X_{21}-derived siRNAs are targeted against the 3' end of the Y transcripts, the secondary siRNAs are most probably produced from this end. Consistently, some $X_{21}Y_{500}Z_c$ plants already show efficient suppression of *CAT* at 4 weeks, but have intermediate GUS activities at 5 weeks. However, after 8 weeks, all $X_{21}YZ_c$ plants exhibit efficient *gus* silencing, indicating that RDR6 can overcome a length of at least 500 nt to give rise to *gus* siRNAs targeting Z_c. This observation is consistent with previous reports in *Nicotiana benthamiana* that demonstrated that VIGS can spread in 3' to 5' direction at least through 332 nt (Vaistij *et al.*, 2002; Petersen and Albrechtsen, 2005). When transgenic *gus*-expressing plants were inoculated with potato virus X (PVX) carrying the 3' fragment of the *gus*-coding sequence (termed S), siRNAs originating from the middle part of *gus* (termed U) were detected, whereas silencing induced with PVX-U did not give rise to siRNAs corresponding to the 5' part of *gus* (termed G; Petersen and Albrechtsen, 2005). Therefore it was concluded that 3' to 5' spreading does occur, but only for a limited distance of 600 nt (from S to U). However, we believe that 3' to 5' spreading over longer distances cannot be excluded, because detection of secondary siRNAs was performed one week after PVX-U inoculation, thus leaving the possibility that after a longer period, siRNAs could have spread further along the *gus* mRNA into the G region. Moreover, the G region might not allow transitivity, because it has been shown to be a weak target (English *et al.*, 1996; Braunstein *et al.*, 2002) and it does not give rise to primary siRNAs (Hutvagner *et al.*, 2000).

Independent of the possible distance of 3' to 5' spreading, amplification initiated from the 3' end of the primary targets Y_{98}, Y_{250} and Y_{500} would imply that a larger population of *CAT2* siRNAs is produced than that of *gus* siRNAs. This assumption is consistent with the longer time required to initiate *gus* silencing with the Y_{500} construct compared to the Y_{98} construct, and especially the Y_7 construct (Fig. 2D). This is also in agreement with the decreasing abundance of secondary siRNAs in *C. elegans* in function of the distance from the primary target region (Sijen *et al.*, 2001). However, although all $X_{21}YZ_c$ plants exhibit efficient *gus* silencing after 8 weeks, only some $X_{21}Y_{250}Z_c$ and hardly any $X_{21}Y_{98}Z_c$ plants obtain a strong reduction in CAT activity.

The observation of plants with efficient *gus* silencing but inefficient *CAT* suppression is at first sight rather surprising. Combined with a postulated higher population of *CAT2* siRNAs (see above), this suggests that the threshold level of siRNAs that is sufficient for efficient targeting of *CAT2* transcripts is higher than that of *gus* siRNAs. Another possible explanation could be a differential abundance of the *CAT2* and *gus* target transcripts, as lower levels of *CAT2* transcripts would require higher levels of siRNAs for efficient targeting. It could also be that the

required siRNA threshold levels are the same for both the *gus* transgene and the *CAT2* endogene, but that the secondary *gus* target Z_c participates in the amplification of 'tertiary' *gus* siRNAs (García-Pérez *et al.*, 2004), while the endogenous *CAT2* transcripts were found not to be template for the production of secondary siRNAs (Fig.3C). Although it cannot be excluded that some secondary endogenous *CAT2* siRNAs are formed, the quantity was too low for detection, unlike secondary siRNAs originating from the Y_{500} transcript. Many other studies also could not provide evidence for transitivity along endogenous sequences (Vaistij *et al.*, 2002; Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005), whereas Sanders *et al.* (2002) detected secondary siRNAs originating from the endogenous β-1,3-glucanase (*glb*) gene in tobacco protoplasts and observed silencing of glucanase tester sequences homologous to the *glb* gene. It is very intriguing and unclear why an endogene-derived transcript would not act as template for RDR6, while a transgene-derived transcript can. Indeed, we observed transitivity along the transgenic *CAT2* sequences. Thus, endogenous sequences seem to be protected from transitivity not by inherent features of the sequences themselves, but rather the context in which they are expressed.

Materials and Methods

Constructs

For the cloning of constructs Y_{98}, Y_{250} and Y_{500}, three PCR fragments cat98, cat250 and cat500 were synthesized using the *Arabidopsis* CAT2 gene (At4g35090) as template with one forward primer 5'-CCGGTTAATTAACGGCTTGCCAGCTTCTGTCC-3' in combination with three different reverse primers: 5'-CCGGTTAATTAAGCCCTATCCGACCCACGCAT-3' for cat98, 5'-CCGGTTAATTAAATGCTGAGAAGTATCCAACT-3' for cat250 and 5'-CCGGTTAATTAAAATGAGCAACTTGCTTTCTG-3' for cat500. The PCR fragments were cut with PacI and inserted by ligation into construct Y_7 (Fig. 1; P35S-gus-npt3'chs) containing the full-length gus-coding sequence, also cut with PacI. The sense orientation of the CAT2 inserts was checked via PCR with the forward primer and a primer specific for the gus sequence. All the T-DNA vectors were brought into the Agrobacterium tumefaciens strain C58C1Rif^R (pMP90).

Plant material and crosses

The production of plants harboring the silencing-inducing locus X_{21} and locus Z_c has been described in Chapter 4. Locus X_{21} is an IR transgene locus with two convergently transcribed neomycin phosphotransferase II (*npt*II) reporter genes (P35S-*nptII*-3'*chs*) that show post-transcriptional gene silencing. Single-copy locus Z_c contains a highly expressed *gus* transgene (P35S-*gus*-3'*nos*). Transgenic plants homozygous for the Z_c locus were transformed with the different Y constructs by using floral dip (Clough and Bent, 1998). Seeds of the dipped plants were harvested and sown on K1 medium supplemented with phosphinothricin (10 mg l^{-1}) for selection of Y_{98}, Y_{250} and Y_{500}. Fluorometric GUS assay and DNA gel blot analysis of the T-DNA integration pattern were performed to select three primary

transformants with a high GUS activity and single-copy locus of Y_{98}, Y_{250} or Y_{500}, excluding *in cis* and/or *in trans* silencing of the *gus* transgenes in Y and Z_c. Progeny plants of these primary transformants were crossed with either transgenic plants homozygous for the X_{21} locus or wild-type *Arabidopsis* (ecotype Columbia). The resulting hybrid seeds were grown on medium selective for the presence of Y_{98}, Y_{250} or Y_{500}. After three weeks they were transferred to soil and grown further under a light regime of 16 h day/8 h night, at 21°C. Seeds of self-fertilized hybrid plants were harvested and sown on medium selective for the presence of either all three loci X_{21}, Y_{98}, Y_{250} or Y_{500}, and Z_c, or for Y_{98}, Y_{250} or Y_{500}, and Z_c. As locus X_{21} and locus Z_c are located on the same chromosome (data not shown), the resulting progeny plants, referred to as $X_{21}YZ_c$, are hemizygous for X_{21} and Z_c and either homozygous or hemizygous for Y. The progeny YZ_c plants contain both loci in either homozygous or hemizygous conditions. The selected progeny plants were transferred to soil after 3 weeks and grown under a light regime of 16 h day/8 h night, at 21°C. Transitive silencing in these plants was studied at different time points after sowing.

Fluorometric GUS assay

Five, 6 and 8 weeks after sowing on selective medium, protein extracts were prepared and GUS activity was measured a as described by Van Houdt et al. (2003).

Catalase activity assay

Four, 7 and 8 weeks after sowing, protein extracts were prepared from two rosette leaves, frozen in liquid nitrogen, by grinding in 120 µl extraction buffer (60 mM Tris-HCl (pH 6.9), 1 mM PMSF, 10 mM DTT, 20% glycerol). The homogenate was centrifuged (15,000x*g*) twice at 4°C for 10 min to remove insoluble material. The supernatant was used for spectrophotometric CAT analysis according to Clare et al. (1984) after determining the total amount of soluble protein with the BioRad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard.

Small RNA analysis

Small RNAs were detected as described by Van Houdt et al. (2003) with minor changes. The enriched low-M_r RNA fraction (35 µg) was loaded on gel, together with RNA oligomers as size controls. To detect *gus* siRNAs, a restriction fragment comprising the full-length *gus*-coding sequence was used, whereas for the *CAT2*-specific siRNAs, two different PCR fragments were utilized: 'CAT250', corresponding to the *CAT2* insert present in Y_{250} and 'CAT250 upstream' covering 398 nt of the *CAT2* sequence located upstream of this insert. ^{32}P-labeled probes were synthesized with the 'Rediprime™ II random prime labelling system' (GE-Healthcare, Little Chalfont, UK) with [α-^{32}P]dCTP. After removal of unincorporated [á-^{32}P]dCTPs with the Bio-Spin p30 Column (BIO-RAD Laboratories, Hercules, CA), the probe was hydrolyzed into fragments of approximately 50 nt. Hybridization and washes were performed as described at 50°C. Labeled membranes were exposed to a PhosphorImager screen (GE-Healthcare). Predominantly, ethidium bromide-stained species of low-M_r RNA, separated by agarose gel electrophoresis (1 µg per lane) were used as loading controls.

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Chapter 6:

Influence of viroid sequences on the spreading of silencing induced by an inverted repeat locus

Submitted for publication:

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Influence of viroid sequences on the spreading of silencing induced by an inverted repeat locus

Abstract

In *Nicotiana tabacum*, spreading of silencing was not observed during viroid-induced silencing, suggesting that transitivity could be blocked by secondary structures present in the target sequence. Here, we demonstrate that insertion of viroid sequences into a primary target Y between the 3' end initially targeted by the silencing inducer X and the upstream region homologous to a secondary target Z, does not prevent transitive silencing of Z in *Arabidopsis thaliana*. This indicates that the induction of transitivity is not influenced by the presence of secondary structures, but rather depends on the nature of the silencing trigger or derived primary siRNAs.

Introduction

Spreading of silencing outside the region originally targeted by a silencing trigger is called transitivity. RNA molecules that are targeted by primary siRNAs can act as substrates for RDR-mediated production of dsRNA, which is subsequently cleaved into secondary siRNAs targeting additional homologous RNAs. Many studies have demonstrated spreading of silencing along transgene sequences (Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005; Petersen and Albrechtsen, 2005; Vaistij *et al.*, 2002), but so far there is only one report about spreading along endogenous sequences (Sanders *et al.*, 2002).

Several silencing inducers have been shown to trigger transitivity: dsRNA molecules (Alder *et al.*, 2003; Sijen *et al.*, 2001), hairpin constructs (Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005), siRNAs (Klahre *et al.*, 2002; Vanitharani *et al.*, 2003), sense transgene loci (Sanders *et al.*, 2002; Van Houdt *et al.*, 2003) and viruses (Braunstein *et al.*, 2002; Petersen and Albrechtsen, 2005; Vaistij *et al.*, 2002). On the other hand, viroids do not induce spreading along target sequences (Vogt *et al.*, 2004). Potato spindle tuber viroid (PSTVd)-induced silencing did not spread along a fusion transgene between the green fluorescent protein (*gfp*) and PSTVd98 (98 nt in length), because neither *gfp*-specific siRNAs could be detected nor silencing of a viroid-free *gfp* transgene was observed in the presence of the silenced *gfp*-PSTVd98 transgene. Viroids are self-complementary RNA molecules that can form a variety of secondary structures (Gross and Riesner, 1980), therefore, it was speculated that these structures could block RDR-mediated dsRNA synthesis (Vogt *et al.*, 2004).

Previously, transitive silencing in plants has been demonstrated in two systems that contain three loci (X, Y and Z) that exhibit a stepwise sequence homology (Van Houdt *et al.*, 2003; Chapters 3 and 4). The silencing-inducing locus X does not only silence the primary target Y, but also triggers transitivity along these target RNAs, resulting in silencing of the secondary target Z. Here, we analyzed the occurrence of transitive silencing of Z when in the primary target Y the 3' end targeted by the silencing inducer X is separated from the upstream region homologous to Z by the same PSTVd fragment used by Vogt *et al.* (2004). We found that transitivity is not influenced by the presence of PSTVd sequences, suggesting that the viroid-induced RNA silencing (VdIRS) system itself prevents spreading of silencing.

Results and discussion

To investigate whether inhibition of transitivity is possibly due to some structural features of particular sequences, such as PSTVd sequences, or to the VdIRS system, we analyzed the

occurrence of transitive silencing in the XYZ silencing system using a primary target Y containing the 98-bp PSTVd cDNA fragment (PSTVd98) postulated to block spreading (Vogt *et al.*, 2004). This fragment was inserted in sense and antisense orientation into construct Y_7 (Fig. 1; P35S-*gus*-*npt*II3'*chs*; Chapter 4), between the *npt*II-3'*chs* region initially targeted by the silencing trigger X_{21} (P35S-*npt*II-3'*chs*) and the full-length *gus*-coding sequence homologous to secondary target Z_c (P35S-*gus*-3'*nos*), providing constructs Y_{Ps} and Y_{Pas}, respectively. As control for the effect of inserting a 98-bp fragment into the primary target, the vector Y_{Cs} was constructed by cloning a 98-bp fragment of the *A. thaliana CHS*-coding sequence with the same GC content, in sense orientation in Y_7. For the three constructs, $X_{21}YZ_c$ and YZ_c plants were obtained by floral dip transformation and crossing experiments (see "Materials and Methods").

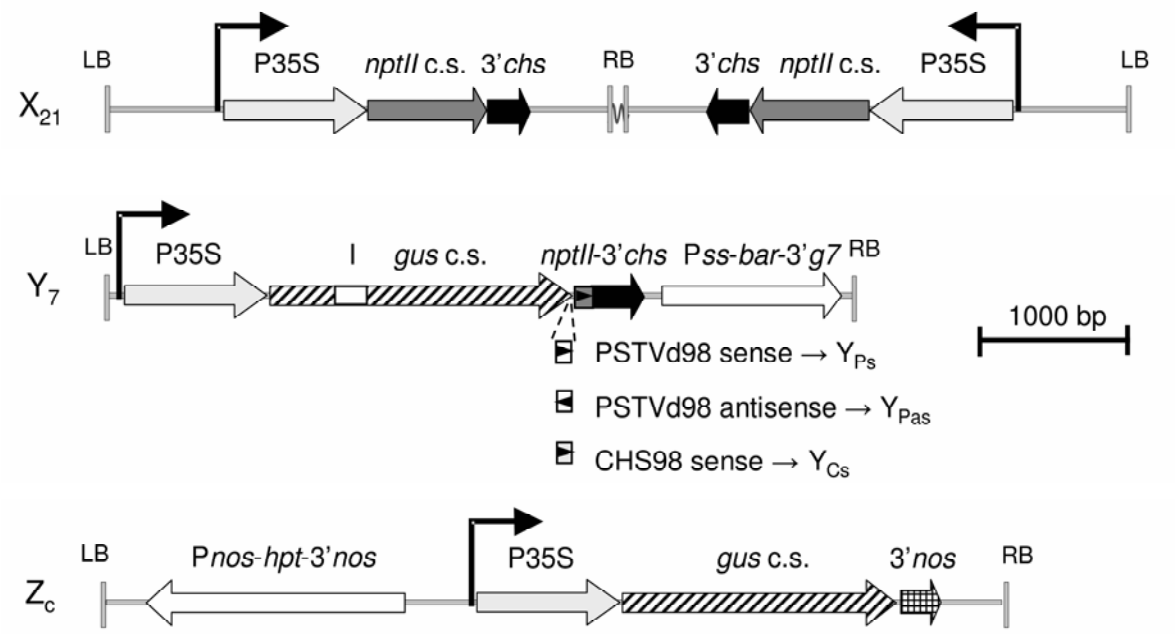

Figure 1. Schematic outline of the T-DNA constructs (drawn to scale), present in locus X_{21}, primary targets Y_{Pas}, Y_{Ps} and Y_{Cs}, and secondary target Z_c. The silencing-inducing locus X_{21} contains two T-DNA copies that are arranged as an IR around the right border. The Y_{Ps}, Y_{Pas} and Y_{Cs} loci and locus Z_c are single copy loci. The PSTVd98 (in sense and antisense orientation) and CHS98 (in sense orientation) fragments are inserted in Y_7 between the *npt*II-3'*chs* region targeted by the silencing trigger X_{21} and the full-length *gus*-coding sequence (*gus* c.s.) homologous to the secondary target Z_c, giving rise to Y_{Ps}, Y_{Pas} and Y_{Cs}, respectively. 3'*chs*, 3'-UTR of the *CHS* gene of *Antirrhinum majus*; 3'g7, 3'-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7; 3'*nos*, 3'-UTR of the nopaline synthase gene; *bar*, bialaphos acetyltransferase coding sequence conferring phosphinothricin resistance; *hpt*, hygromycin phosphotransferase coding sequence; I, artificial intron; *npt*II c.s., neomycin phosphotransferase II coding sequence; P35S, CaMV 35S promoter; P*nos*, nopaline synthase promoter; P*ss*, promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase; LB, left T-DNA border; RB, right T-DNA border.

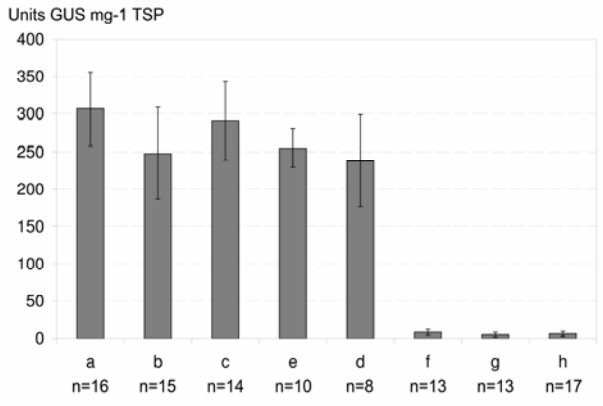

Figure 2. GUS activity levels (in Units GUS mg^{-1} TSP) in protein extracts of leaf tissue harvested from $Y_{Ps}Z_c$ (a), $Y_{Pas}Z_c$ (b), $Y_{Cs}Z_c$ (c), Z_c (d), $X_{21}Z_c$ (e), $X_{21}Y_{Ps}Z_c$ (f), $X_{21}Y_{Pas}Z_c$ (g), and $X_{21}Y_{Cs}Z_c$ (h) plants. SD is given by error bars. n, total number of plants tested; TSP, total soluble protein.

Figure 2 shows the mean GUS activity measured in leaf protein extracts from hybrid $X_{21}YZ_c$ and YZ_c plants and control Z_c and $X_{21}Z_c$ plants. In all YZ_c plants (Fig. 2 a,b and c), GUS activity was as high as in the control plants (Fig. 2 d and e), whereas in all $X_{21}YZ_c$ plants (Fig. 2 f, g and h) it was less than 5% of the mean GUS value of the corresponding YZ_c plants (data not shown). Based on this observation, we can conclude that the insertion of a fragment of 98 bp between the directly targeted and adjacent regions in Y does not interfere with the spreading of silencing. Moreover, insertion of the PSTVd98 fragment does not block transitive silencing of Z_c, indicating that the failure of PSTVd infection to induce transitivity (Vogt *et al.*, 2004) is not caused by some secondary structure formed by the PSTVd sequence present in the target transcripts, as speculated, but is specific for the VdIRS system. Several explanations could account for the conflicting results. First is the differential involvement of RDR6 in the production of the triggering dsRNA. In the XYZ system, the IR transgene locus X_{21} is thought to produce aberrant transcripts as a result of disturbed transcription near the centre of the IR that might form secondary structures. These aberrant RNAs (abRNAs) are postulated to act as substrates for RDR6-dependent dsRNA synthesis (Vaistij *et al.*, 2002). Consistently, IR loci have been shown to require RDR6 to induce posttranscriptional gene silencing (PTGS) in *A. thaliana* (Butaye *et al.*, 2004). In the VdIRS system, the triggering PSTVd dsRNA molecules are replication intermediates that are produced in the nucleus by the DNA-dependent RNA polymerase II via a rolling-circle mechanism (Schindler and Muhlbach, 1992). However, hairpin constructs also induce transitivity (Himber *et al.*, 2003; Kościańska *et al.*, 2005; Miki *et al.*, 2005), although RDR6 is not required for hairpin-induced silencing (Béclin *et al.*, 2002).

A second explanation could be a differential preference for the RNA-induced silencing complex (RISC)-dependent or the RDR/Dicer-dependent pathways for target RNA degradation (Sanders *et al.*, 2004). In the XYZ system, it is conceivable that the population of X_{21}-derived

siRNAs is rather smal, because dsRNA molecules are produced from abRNAs that arise sporadically compared to the abundant normal mRNAs. Moreover, these siRNAs have been shown to enter the RDR/Dicer pathway, because a consistently larger accumulation of *chs*-specific siRNAs was observed in plants that contained both a silencing-inducing IR locus X and a primary target Y compared to those harboring only locus X (Van Houdt *et al.*, 2003), and transitive silencing of a secondary target could be obtained (Chapters 3 and 4). During viroid infection, a larger population of PSTVd dsRNAs might be produced, resulting in very efficient RISC-mediated degradation of GFP-PSTVd98 target RNAs that are also candidate template RNA molecules for RDR6. As a consequence, secondary *gfp*-specific siRNAs are either not produced at all or in an amount insufficient to induce silencing of the viroid-free *gfp* transgene. However, hairpin-derived siRNAs are also believed to be very abundant and they nevertheless induce transitivity, although this might be observed in transformants with intermediate expression of the hairpin construct. A third possibility is the different target regions of the trigger-derived siRNAs. In the XYZ system, the X_{21}-derived siRNAs are directed against the 3' end of primary target Y, downstream of the PSTVd98 fragment, whereas in the VdIRS system, PSTVd-specific siRNAs target the PSTVd98 fragment itself. Perhaps RDR6 could overcome the secondary structure possibly formed by the PSTVd98 fragment by the action of an RNA helicase (SDE3), but might be unable to initiate dsRNA synthesis starting within this structure. A fourth explanation could be a differential susceptibility of the target RNAs to RDR6-mediated siRNA amplification. Indeed, it is striking that many endogene-derived mRNAs seem to be inefficient substrates for RDR6, but what distinguishes endogene RNAs from transgene RNAs remains unclear. A fifth difference is the differential DNA methylation of the target sequences. In the XYZ system, spreading of methylation into the sequences that are not homologous to the silencing-inducing IR locus is observed (data not shown; Van Houdt *et al.*, 2003; Chapter 3), whereas during viroid-induced silencing only the PSTVd-specific sequences become fully methylated (Wassenegger *et al.*, 1994). Finally, we cannot exclude the possibility that the use of different plant species (*Arabidopsis* versus tobacco) accounts for the conflicting observations.

Materials and Methods

Constructs

For the cloning of constructs Y_{Pas}, Y_{Ps} and Y_{Cs}, the PSTVd98 fragment and 98 nt of the *CHS*-sequence (CHS98) were amplified by PCR using primers containing a *Pac*I site: 5'-CCGGTTAATTAACCGCTTTTTCTCTATCTA-3' and 5'-CCGGTTAATTAATGAACCACAGGAACCACG-3' for PSTVd98 and 5'-CCGGTTAATTAAGAGAGGAACGCTGTGCAAGA-3' and 5'-CCGGTTAATTAAGTGTGGCCACGACAGGAGAA-3' for CHS98. As templates we used the construct 702GFP98 {described in Vogt *et al.* (2004)} for PSTVd98, and vector

pPs35SCHS800npt3'chs, harbouring the last 800 nt of the *A. thaliana CHS*-coding sequence, for CHS98. The PCR-fragments were cut with *Pac*I and inserted into the unique *Pac*I site in construct Y_7 (P35S-*gus*-*npt*II-3'*chs*) containing the full length *gus*-coding sequence {described in Chapter 4}. The orientation of the inserts was checked via PCR and sequencing. Constructs Y_{Pas} and Y_{Ps} with the PSTVd98 fragment in sense and antisense orientation, respectively, and Y_{Cs} with the CHS98 fragment in sense orientation (Fig. 1), were introduced in the *Agrobacterium tumefaciens* strain C58C1RifR (pMP90).

Plant material and crosses

The production of plants harboring the silencing-inducing IR transgene locus X_{21} (P35S-*npt*II-3'*chs*) and locus Z_c (P35S-*gus*-3'*nos*) has been described previously (Chapter 4). Transgenic plants homozygous for the Z_c locus were transformed with the floral dip method (Clough and Bent, 1998). Seeds of the dipped plants were harvested and sown on K1 medium supplemented with phosphinotricin (10 mg l^{-1}) for selection of Y_{Pas}, Y_{Ps} and Y_{Cs}. Fluorometric GUS assay and DNA gel blot analysis of the T-DNA integration pattern were performed to select three primary transformants with highly expressed single copy loci Y_{Pas}, Y_{Ps} and Y_{Cs}, excluding *in cis*- and *in trans*-silencing of the *gus* transgenes. Progeny plants of these primary transformants were crossed with either transgenic plants homozygous for the X_{21} locus or wild-type *Arabidopsis* (ecotype Columbia). The resulting hybrid seeds were grown on medium selective for the presence of Z_c. After 17 days, the presence of Y_{Pas}, Y_{Ps} and Y_{Cs} was checked by a callus induction test on leaves of the seedlings (De Neve *et al.*, 1997). After two weeks, the $X_{21}YZ_c$, $X_{21}Z_c$, YZ_c and Z_c plants, all hemizygous for the present loci, were transferred to soil and grown further under a light regime of 16 h day/8 h night, at 21°C. The GUS activity was determined after 5 weeks. After two weeks they were transferred to soil and grown further under a light regime of 16 h day/8 h night, at 21°C. The GUS activity was determined after 5 weeks.

Enzymatic assay

Protein extracts were prepared and GUS activity was measured as described by Van Houdt *et al.* (2000).

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Chapter 7:

Summary

Summary

RNA silencing can be triggered upon introduction of transgenic DNA by the intentional or unintentional production of dsRNA from the transgene(s). In general, RNA silencing is a two-step process. In the first step, the triggering dsRNA molecules are processed into 21 to 26-nt siRNAs by an RNase III-like enzyme Dicer or Dicer-like. The second step, the sequence-specific cleavage of cognate target RNAs, is brought about by siRNAs bound to specific proteins, which together form a multi-component nuclease, RISC. The Dicer/RISC pathway accounts for the initiation of the silencing process and the homology-dependent selection and degradation of target RNAs. However, plants, fungi and nematodes have developed an additional RDR/Dicer-mediated amplification pathway that ensures a robust RNA silencing response by the production of secondary siRNAs from regions outside of the sequence initially targeted by trigger-derived primary siRNAs. These secondary siRNAs are able to induce silencing of additional targets without homology to the initial silencing trigger, which is called transitive silencing. The studies described in this thesis give a proof of concept of transitivity as alternative silencing technology and contribute to a better understanding of the mechanism of transitive silencing.

In chapter 3, we investigated in *Nicotiana tabacum* whether a post-transcriptionally silenced invertedly repeated transgene locus X is able to silence a non-homologous transgene Z, when a stepwise homology is created by introducing a chimeric primary target Y that has one region homologous to the silencing inducer X and a second upstream region homologous to the secondary *gus* target Z. We found that *in trans*-silenced Y transcripts produced secondary *gus*-specific siRNAs that were mainly derived from the 3' part of the *gus* transcripts, guiding the degradation of Z-derived transcripts. This indicates that silencing can spread in 3' to 5' direction, but is limited to the 3' end of the target RNA (about 1000 nt). Moreover, the region that gave rise to the secondary siRNAs became methylated upon silencing. We also demonstrated that a transcriptionally silenced epiallele of locus X that was generated after *in vitro* propagation in callus culture was heavily methylated in the promoter region, in contrast to the post-transcriptionally silenced epiallele.

In chapter 4, we developed a similar XYZ system in *Arabidopsis thaliana* to assess whether a transitive silencing signal is also able to trigger silencing of an endogenous target, namely the *CAT2* gene, which is part of a gene family (*CAT1*, *CAT2* and *CAT3*). We found that secondary siRNAs could down-regulate catalase expression, but the efficiency of silencing depended on the zygosity of the primary target locus and to a lesser extent on that of the silencing inducer. This suggests that the RDR6-mediated production of secondary siRNAs is limited by the input of

candidate template RNAs, which is influenced by RISC-mediated degradation. The *CAT1* and *CAT3* genes, which have a sequence identity with *CAT2* of 78% and 75%, respectively, were also down-regulated, but in a different degree, which is probably due to their different expression levels.

In chapter 5, we studied the effect of inserting in primary target Y *CAT2* fragments with increasing lengths (98 nt, 250 nt and 500 nt) between the 3' end targeted by the silencing inducer X and the upstream region homologous to the secondary *gus* target Z on the silencing response. Temporal analysis of the GUS activity demonstrated that the silencing efficiency was positively correlated with the amount of secondary *gus*-specific siRNAs and increased through time until all XYZ plants showed full suppression of the *gus* transgene after 8 weeks. This indicates that silencing gradually spreads in 3' to 5' direction over a distance of at least 500 nt. The plant-to-plant variation in the onset of efficient *gus* silencing appeared to be negatively influenced by increasing the distance between the directly targeted and adjacent regions in primary target Y. Temporal analysis of the catalase activity also showed an increase in silencing efficiency through time, which was positively correlated with the length of sequence homology between primary and secondary target, but only the primary target with a *CAT2*-insert of 500 nt was able to induce an efficient catalase silencing response in all plants after 8 weeks. This suggests that the amplification of siRNAs is not an endless, self-perpetuating process, but gradually produces an expanding population of secondary siRNAs until a certain steady-state level is reached. Whether this steady-state level is able to exceed the threshold for efficient silencing seems to depend not only on the length of sequence homology between primary and secondary target, but also on the nature and possibly the stability of the primary target transcripts that act as substrate for RDR6 (chapters 4 and 5).

In chapter 6, we demonstrated that the presence of potato spindle tube viroid (PSTVd) sequences in a primary target Y did not interfere with transitive silencing of a transgenic target Z in *A. thaliana*, which is in contrast to the observation that PSTVd-induced silencing of a transgene did not induce spreading of silencing in tobacco (Vogt *et al.*, 2004). This suggests that the inhibition of transitivity is specific for the viroid-induced silencing system, and not caused by some structural feature of the PSTVd sequence in the primary target.

Chapter 8:

Samenvatting

Samenvatting

RNA silencing kan worden uitgelokt door de introductie van transgeen DNA door de gewenste of ongewenste productie van dubbelstrengig RNA (dsRNA) door het transgen. In het algemeen bestaat RNA silencing uit twee stappen. In de eerste stap worden de inducerende dsRNA-molecules verwerkt tot 21-26 nt siRNA's door een RNase III-gelijkend enzym, Dicer of Dicer-like. De tweede stap, de sequentie-specifieke verknipping van doelwit RNA's, wordt gemedieerd door siRNA's die gebonden zijn met specifieke proteïnen, die samen een multi-component nuclease, het RISC, vormen. De Dicer/RISC reactieweg staat in voor de initiatie van het silencing proces en de homologie-afhankelijke selectie en degradatie van doelwit RNA's. Echter, planten, fungi en nematoden hebben een additionele RDR/Dicer-gemedieerde amplificatie reactieweg ontwikkeld die een robuste RNA silencing respons verzekert door de productie van secundaire siRNA's afkomstig van regio's die naast de oorspronkelijke doelwitregio gelegen zijn. Deze secundaire siRNA's zijn in staat silencing te induceren van additionele doelwitten die geen homologie vertonen met de oorspronkelijke silencing trigger, wat transitieve silencing genoemd wordt. De studies beschreven in dit proefschrift geven een 'proof of concept' van transitiviteit als een alternatieve silencing technologie en dragen bij tot een beter inzicht in het mechanisme van transitieve silencing.

In hoofdstuk 3 onderzochten we in *Nicotiana tabacum* of een post-transcriptioneel gesilencet transgeen locus X bestaande uit een omgekeerde herhaling, de expressie van een niet-homoloog transgen Z drastisch kan verlagen, wanneer een stapsgewijze homologie gecreëerd wordt door de introductie van een chimeer primair doelwit Y, bestaande uit een regio homoloog met de silencing-inducer X en een tweede stroomopwaartse regio homoloog met het secundaire *gus*-doelwit Z. We vonden dat *in trans*-gesilencete Y transcripten secundaire *gus*-specifieke siRNA's produceerden die voornamelijk afkomstig waren van het 3' gedeelte van de *gus*-transcripten en die de degradatie van transcripten afkomstig van Z bewerkstelligden. Dit duidt aan dat silencing kan uitbreiden in 3' to 5' richting, maar beperkt is tot het 3' gedeelte van het doelwit RNA (ongeveer 1000 nt). Bovendien werd de regio die aanleiding gaf tot secundaire siRNA's gemethyleerd na silencing. We toonden ook aan dat een transcriptioneel gesilencet epiallel van locus X, dat ontstond na *in vitro* propagatie via callus culturen, sterk gemethyleerd was in de promoter-regio, in tegenstelling tot het post-transcriptioneel gesilencete epiallel.

In hoofdstuk 4 ontwikkelden we een gelijkaardig XYZ systeem in *Arabidopsis thaliana* om na te gaan of een transitief silencing signaal ook neerregulatie van een endogeen doelwit kan uitlokken, namelijk van het *CAT2* gen, dat deel is van een genfamilie (*CAT1*, *CAT2* en *CAT3*). We konden aantonen dat secundaire siRNA's in staat waren de catalase-expressie te

reduceren, maar de silencing efficiëntie was afhankelijk van de zygotie van het primaire doelwit-locus, en in mindere mate dat van de silencing-inducer. Dit suggereert dat de RDR6-gemedieerde productie van secundaire siRNA's beperkt is door de toevoer van kandidaat template RNA's, wat beïnvloed wordt door RISC-gemedieerde degradatie. De *CAT1* en *CAT3* genen, die een sequentie-identiteit met *CAT2* hebben van 78% en 75%, respectievelijk, worden ook neergereguleerd, maar in verschillende mate, wat waarschijnlijk komt door de verschillende expressieniveaus.

In hoofdstuk 5 bestudeerden we hoe het toevoegen van *CAT2*-fragmenten met toenemende lengtes (98 nt, 250 nt en 500 nt) in een primair doelwit Y, tussen het 3' einde dat getarget wordt door de silencing-inducer X en het stroomopwaartse gebied homoloog met het secundaire *gus*-doelwit Z, effect heeft op de silencing respons. Tijdsanalyse van de GUS activiteit toonde aan dat de silencing efficiëntie positief gecorreleerd was met de hoeveelheid van secundaire *gus*-specifieke siRNA's en toenam in de loop van de tijd totdat alle XYZ planten volledige suppressie van het *gus*-transgen vertoonden na 8 weken. Dit duidt aan dat silencing gradueel uitbreidt in 3' naar 5' richting over een afstand van minstens 500 nt. De plant-tot-plant variatie in de initiatie van efficiënte *gus*-silencing bleek negatief beïnvloed te worden door een toenemende afstand tussen de primaire en secundaire doelwitregio's in het primaire doelwit Y. Tijdsanalyse van de catalase activiteit toonde ook een toename in de silencing efficiëntie in de loop van de tijd, wat positief gecorreleerd bleek te zijn met de lengte van de sequentie-homologie tussen primair en secundair doelwit. Echter, enkel het primaire doelwit met een *CAT2*-fragment van 500 nt kon een efficiënte catalase silencing respons uitlokken in alle planten na 8 weken. Dit suggereert dat de amplificatie van siRNA's geen eindeloos, zelf-onderhoudend proces is, maar geleidelijk een toenemende populatie van secundaire siRNA's vormt tot een bepaald steady-state niveau bereikt is. Of dit steady-state niveau in staat is om de drempel voor efficiënte silencing te overschrijden, lijkt afhankelijk te zijn van niet enkel de lengte van sequentie-homologie tussen primair en secundair doelwit, maar ook op de aard en waarschijnlijk de stabiliteit van de primaire doelwit-transcripten die fungeren als substraat voor RDR6 (hoofdstukken 4 en 5).

In hoofdstuk 6, toonden we aan dat de aanwezigheid van 'potato spindle tube viroid' (PSTVd)-sequenties in een primair doelwit Y niet interfereerden met transitieve silencing van een transgeen doelwit Z in *A. thaliana*, wat in contrast is met de waarneming dat PSTVd-geïnduceerde silencing van een transgen geen verspreiding van silencing in tabak uitlokte. Dit suggereert dat de inhibitie van transitiviteit specifiek is voor het viroid-geïnduceerde silencing systeem, en niet veroorzaakt wordt door een bepaalde structurele eigenschap van de PSTVd-sequentie aanwezig in het primaire doelwit.

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